

COLLAGEN AND BLEEDING TIME IN EHLERS-DANLOS SYNDROME AND IN SCOLIOSIS

by
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Abstract		A4	A5
A method for quantification of collagen-induced platelet aggregation is described. Native collagen in suspensions prepared by direct homogenization of freeze-dried fascia probably retains more of the original platelet aggregating properties than suspensions prepared from chemically well defined solutions of collagen. The <u>in vitro</u> values recorded varied with the anatomical structure from which the biopsy specimens were obtained and the platelet-rich plasma by which the test was performed. The variation was reduced to acceptable level by standardization of the various steps of the method. There is a correlation between the <u>in vitro</u> platelet aggregation potency of collagen and the <u>in vivo</u> determined bleeding-time performed according to a sensitive modification of Ivy's method. The collagen from 7 patients with the Ehlers-Danlos syndrome had normal platelet aggregating properties and the bleeding time (Ivy) was only slightly prolonged. There were three patients from a family with type I of the syndrome (gravis type), three from a family with type III (benign and hypermobile type) and one patient with an unclassifiable type of the syndrome. Despite their bleeding tendency all had been operated on several times without any bleeding complication. In idiopathic scoliosis the platelet aggregating potency of the collagen was decreased and suspensions made from fascia contained an increased amount of collagen, which can be caused by an altered structure of the connective tissue. The collagen in two out of four cases of congenital scoliosis showed similar abnormalities. The alteration of the collagen was also probably reflected in a slightly prolonged bleeding time, which is of no clinical importance but which may elucidate the role of collagen in the etiology of idiopathic scoliosis which appears to be multifactorial. Collagen may play a role not only in the etiology of idiopathic scoliosis, but also in paralytic and congenital scoliosis, in which similar abnormalities were found.			
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TO MY FAMILY

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- I. Udén, A. and Nilsson, I. M. : A METHOD FOR MEASURING COLLAGEN-INDUCED PLATELET AGGREGATION. *Thrombosis Research* 12:1087-1097, 1978.
- II. Udén, A. and Nilsson, I. M. : CORRELATION BETWEEN THE BLEEDING TIME AND THE PLATELET AGGREGATING ACTIVITY OF COLLAGEN IN SCOLIOSIS *Thrombosis Research* 16:93-95, 1979.
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- VI. Udén, A., Nilsson, I. M. and Willner, S. : BLEEDING TIME IN SCOLIOSIS. Submitted for publication.

These articles will be referred to by their Roman numerals in the text.

INTRODUCTION

Collagen is the most abundant protein in the human body. This fibrous protein is responsible for practically all the tensile strength of the connective tissue. The tensile strength of a collagen fibre 1 mm in diameter is 10-40 kp (Grant and Prockop 1972a). In recent years collagen has been the subject of very wide research, which has substantially contributed to our knowledge of its structure and function in health and in disease.

The following survey of fundamental data on the structure and biosynthesis of collagen relevant to the discussion of various aspects of the subject in this thesis, is based on some published reviews (Grant and Prockop 1972a, b, c. Kivirikko and Risteli 1976, Miller 1976, Prockop et al. 1979a, b).

The collagen molecule

The collagen molecule is built up of three polypeptide chains called alpha-chains. Each of these chains contains about 1000 amino-acid-residues and is coiled into a left-handed helix with about three amino-acids per turn. The three helical alpha-chains are then twisted around each other into a right-handed super helix to form a rigid structure. This triple helical molecule is about 300 nm long and 1.5 nm in diameter, and in the second level of organization the molecules are arranged laterally and longitudinally to form micro-fibrils and fibres.

With the exception of the short non-helical sequences at the ends of the alpha-chain every third amino-acid is glycine. The polypeptide chain can therefore be described as a polymer of tripeptides (xy - gly)_n.

The presence of glycine, the smallest amino-acid in every third position is essential since it occupies the limited space in the centre of the triple helix. About 100 of the amino residues in the x-position are proline and about 100 of them in the y-position are hydroxyproline. Both are rigid cyclic amino-acids and therefore limit the rotation of the polypeptide and thus contribute to the stability of the triple helix. Two thirds of the x- and y-

positions are occupied by other amino-acids, which tend to be clustered in groups of hydrophobic and charged residues, and since their side chains extend from the centre of the triple helix they determine how the collagen molecules associate with each other. They are packed so that each molecule is longitudinally displaced somewhat less than one quarter of its length (quaternary structure of collagen).

The biosynthesis and cross-linking of collagen

The initial polypeptide synthesized is called pro- α -chain, which contains peptide extensions at both ends; the amino-terminal is about 15 nm long and the carboxyterminal about 10 nm. This polypeptide undergoes extensive post-translational modifications and the general steps are shown in Table 1. from Kivirikko and Risteli (1976).

Table 1. General steps in the biosynthesis of collagen.^{x)}

<u>Biosynthetic step</u>	<u>Biological significance of the biosynthetic step</u>
1. Transcription and translation	Primary structure
2. Hydroxylation of prolyl residues	Essential for triple helix formation at 37°C
3. Hydroxylation of lysyl residues	Essential for glycosylation reactions Essential for stability of cross-links
4. Glycosylations of hydroxylysyl residues	Possibly affect fibre formation
5. Chain association and disulphide bonding	Essential for triple helix formation
6. Triple helix formation	Essential for normal rate of procollagen secretion into the extracellular matrix Several properties of the molecule
7. Secretion of procollagen into the extra-cellular matrix	Essential for extra-cellular modifications
8. Conversion of procollagen into collagen	Essential for normal fibre formation
9. Aggregation of collagen molecules	Fibre formation
10. Cross-link formation	Essential for stability of fibres

^{x)} These steps are not entirely sequential. For instance, steps 2-4 are initiated while the polypeptide chains are growing on the ribosomes and they are continued after the release of completed polypeptide chains from the ribosomes. Also, in certain instances, step 5 can occur before steps 2-4.

The prolyl residues in the γ -position are hydroxylized to hydroxyproline by a prolyl hydroxylase. Ferrous ions, molecular oxygen, α -ketoglutarate and ascorbic acids are co-factors. There are specific requirements of the substrate; the residue must be in the γ -position and the peptide must be non-helical, thus the enzyme does not act on collagen that is triple helical. Each α -chain must have about 90 residues that are hydroxylated to form a triple helix that is stable at 37°C . On the other hand, after the chains have acquired the necessary hydroxyproline and the inter-chain disulphide bonds in the pro-collagen peptides necessary for helix formation, they fold into a triple helix and no further hydroxylation is possible. This implies that the amount of hydroxyproline in collagen is relatively constant. Since hydroxyproline occurs virtually only in collagen, collagen can be determined from the amount of hydroxyproline in solutions and in specimens.

Lysyl residues are hydroxylized by another enzyme-lysyl-hydroxylase - which has the same co-factors as prolyl-hydroxylase. The hydroxylation must also occur before the helix is formed. Sugar residues can be added to the hydroxylysyl residues. Galactose can be added to the hydroxylysyl residue by a galactosyl transferase, and glucose can be added to the galactosyl hydroxylysyl residues by a glucosyltransferase. These modifications of the lysyl residues are essential for intermolecular cross-linking.

The function of the aminoterminal and the carboxyterminal propeptides is not known. Five possibilities have been suggested:

The propeptides may prevent premature fibril formation, help to direct assembly of the protein into fibrils, direct association of pro- α -chains so that each molecule will contain the correct three pro- α -chains, increase the rate and efficiency of folding the pro- α -chain into a triple helix, or, after they are cleaved from the molecule they may function in a feed-back mechanism to control the amount of procollagen synthesized by the cells. There is evidence in support of each of the functions suggested. Outside the cell the propeptides are split off by at least two enzymes, a procollagen amino-protease that removes the amino-propeptide and a carboxy-protease that removes the carboxy-propeptides.

After the conversion of procollagen to collagen the molecules aggregate in the above mentioned specific way. However, the tensile strength of collagen depends on inter-

molecular cross-links between lysyl and/or hydroxylysyl residues which also can be modified by the sugar residues. A copper-dependent lysyloxidase catalyses the first step, an oxidative deamination of the epsilon-amino groups of certain lysyl and hydroxylysyl residues. This reaction can be inhibited by beta-amino-propionitrile and the collagen then produced lacks tensile strength and is called lathyr collagen since seeds of sweet pea (*Lathyrus odoratus*) contain this copper-chelating agent.

These reactive aldehydes produced by the lysyl oxidase form two major types of cross-links. One is an intramolecular cross-link formed by aldol condensations of two aldehydes. In the other an aldehyde in the non-helical region derived from lysine (allysine) or hydroxylysine (hydroxy allysine) with or without sugar residues reacts with unaltered epsilon-amino groups of lysine or hydroxylysine in the helical region of the molecule.

These reducible cross-links are common in newly synthesized collagen, but they gradually decrease with time and in mature collagen there are no reducible cross-links. The collagen is then insoluble. It has been proposed that the reducible cross-links act only as intermediate cross-links which serve as precursors to a variety of more stable non-reducible cross-links, whose nature we still know very little about, but those derived from deaminated hydroxylysine are considered more stable.

Different types of collagen

There are at least five types of collagen and some of their main characteristics are summarized in Table 2.

Type I is the most abundant collagen in the human body. In the low age groups it is readily extracted as native monomeric molecules from skin and other soft tissues by salt or diluted acid solvents.

Type II-collagen occurs in hyaline cartilage and in some structures in the eye. It is practically insoluble in salt and acid solvents.

Type III-collagen is more abundant in fetal life than in adult life. In adult tissue, such as large arteries, muscle, lung, bowel, liver and skin it accounts for 10-50 % of the total collagen. The fibres are smaller than those of type I-collagen and judging from its

distribution among different tissues, it is suspected that it is suited to function in distensible tissues. It is much less soluble than type I.

Table 2. Different types of collagen (from Prockop et al. 1979a).

Type	Molecular form	Chemical characteristics	Tissue distribution
I	$(\alpha 1(I))_2\alpha 2$	Composed of two kinds of chains. Low in hydroxylysine and glucose.	Bone, tendon, skin, ligament, fascia, dentin, arteries and uterus.
II	$(\alpha 1(II))_3$	Relatively high in hydroxylysine and glucose.	Hyaline cartilage and eye.
III	$(\alpha 1(III))_3$	High in hydroxyproline. Low in hydroxylysine. Contains cysteine.	Skin, arteries and uterus.
IV	$(\alpha 1(IV))_3$	High in hydroxylysine and glucose. May contain large globular regions.	Basement membranes.
V	$(\alpha A \text{ and } \alpha B)$	High in hydroxylysine and glucose. May contain large globular regions.	Basement membranes and maybe other tissues.

Collagen and platelets in hemostasis

Collagen is not only responsible for the tensile strength of connective tissue but also plays an important role in normal hemostasis. As early as 1851 Wharton-Jones showed that "colourless corpuscles" accumulate at points of local damage in blood vessels, and in 1822 Bizzozero recognized the role of platelets (Caen et al. 1977). Bounameaux (1959) and Hugues (1960) showed that the platelets adhered to exposed subendothelial collagen fibrils in the damaged vessel wall. The mechanism of the collagen platelet interaction has been the subject of extensive research. Jamieson et al. (1971) suggested that the adhesion of platelets to collagen was mediated by an interaction between a glucosyltransferase on the surface of the platelets and the sugar residues of the hydroxylysine of the collagen molecule. This hypothesis was based on the fact that inhibitory effects of different substances on both the glucosyltransferase of the platelets and on the platelet-collagen adhesion were equal, that the incorporation of glucose from uridine-diphosphate glucose into collagen during the process of collagen adhesion and that the enzyme was located on the outer surface of the platelet membrane. However, there is now evidence that this hypothesis is untenable. The platelet glucosyltransferase requires non-helical substrate.

The adhesion of platelets to native collagen following endothelial damage can therefore hardly be mediated by this enzyme (Menashi et al. 1976). Also collagen devoid of intact carbohydrate residues is fully capable of inducing platelet aggregation (Santoro and Cunningham 1977).

Fibronectin has been suggested to be the collagen-binding protein of the platelets (Bensusan et al. 1978). Fibronectin, a cold insoluble globulin (CIG), is a glucoprotein of human plasma and occurs also in the connective tissue and other structures (Vaheri et al. 1978). Fibronectin contributes to cell spreading and to adhesion of cells to collagen (Pearlstein 1976, Vaheri and Ruoslahti 1975). The proteins remaining bound to collagen fibres after platelet collagen aggregation and after the removal of the rest of the platelets by sonication were immunologically identified as CIG and also such purified CIG does bind to collagen (Bensusan et al. 1978). It has also been shown that fibronectin is stored in the alpha-granules of the platelets and that it can be released by collagen (Zucker et al. 1979).

The in vitro platelet aggregation

When platelets are exposed to native collagen they aggregate and adenosine diphosphate (ADP) plays a central role in this interaction. ADP, released from the platelets by the collagen, aggregates other platelets leading to the formation of platelet aggregates (Zucker and Borelli 1962, Holmsen et al. 1969, Kattlove and Gomez 1975). This can also be studied in vitro by the method of Born (1962). He prepared platelet-rich plasma (PRP) from human blood to which sodium citrate or heparin had been added to prevent clotting. The blood was centrifuged for 20 minutes at 500 g. The plasma was then used for the study of platelet aggregation. Light with a wave-length of 600×10^{-7} m was passed through a transparent plastic tube with PRP. The optical density proved proportional to the concentration of platelets in it. The PRP was stirred continuously by a small polyethene-coated iron rod which was rotated magnetically. The stirring was continued until the optical density was read. O'Brien et al. (1966) and Mills and Roberts (1967) modified the method and used constant recording of the transmission. The speed of aggregation was calculated from the declination of the curve. When ADP was added there were two phases of aggregation. The first phase started immediately after the addition of ADP and the speed of platelet aggregation was proportional to the amount of ADP added. In the second phase, which is caused by ADP released from the platelets there was a sigmoid relation in the plot of transmission and amount of ADP added (Mills and Roberts 1967). When

collagen was used there was no first phase and there was a lag between the addition and the initiation of the aggregation. The method of Born (1962) has been used in numerous investigations of the platelet aggregating potency of collagen.

THE EHLERS-DANLOS SYNDROME

The Ehlers-Danlos syndrome (EDS) was originally described in 1682 by Job van Meekeren, who reported a case which manifested "extraordinary dilatability of the skin". Ehlers in 1901 and Danlos in 1908 recognized the syndromic association of loose-jointedness, hyper-extensible skin, subcutaneous hemorrhage and subcutaneous pseudotumours (Barabas and Barabas 1967).

Clinical features

The hypermobility of the joints can be extreme allowing remarkable distortions, and luxations of the joints may occur. The skin is often thin and velvety. The hyper-extensibility varies, and in pronounced cases the skin may be pulled out in folds several centimeters without causing any pain. The skin splits easily after even minor everyday trivial injuries. These wounds bleed only little, but they may be difficult to close because sutures are liable to cut through the skin. The scars are often gaping and cigarette-paper thin. Instead of contracting they tend to spread. These scars are pathognomonic and are often diagnostically useful. The scars are common on bony prominences such as the forehead, shins, elbows and kneecaps.

A tendency to ready bruisability with subcutaneous hematoma is common and occasionally gives rise to molluscoid pseudotumours over bony prominences. Subcutaneous spheroids or spherules are frequently seen and can be palpated as small hard freely mobile nodules. They may calcify and be radiographically demonstrable. In some cases wide-set eyes with epicanthal folds, a broad nasal bridge and bat-ears give the face a characteristic appearance (Barabas and Barabas 1967, Beighton 1969, Mc Kusick 1972).

As in other categories of genetic disease, clinical features, heredity and biochemical features have been used to distinguish various forms. Today at least seven forms are recognized (Table III:1).

The variants of the Ehlers-Danlos syndrome

Type I.

In this variety the hyperextensibility of the skin is a prominent feature. The tendency to skin splitting is severe and cigarette-paper thin scars are often seen on the forehead, on the knees and on the elbows. Molluscoid pseudotumours and subcutaneous spheroids are seen in the majority of these patients. Joint hypermobility is generalized and gross musculo-skeletal malformations are common. A varying degree of scoliosis may sometimes develop. The tissue is sometimes unduly friable and difficulties at operation have been reported. The bruising tendency is usually moderate. The patient may have irregular teeth, and epicanthic folds, Varicose veins are very common. There is a high incidence of prematurity due to early rupture of the fetal membranes (Beighton 1969, Beighton and Horan 1969).

Type II.

In this variety the stigmata are the same as in type I, but less obvious and may easily escape detection. Joint hypermobility is limited. The skin is less hyperextensible and, unlike what is seen in type I, the skin has no tendency to split. Tissue friability is not severe and orthopedic complications are not hazardous. The bruising tendency is often only mild. Varicose veins are not common (Beighton 1969, Beighton and Horan 1969).

Type III.

The joint hypermobility is generalized and gross, while the skin hyperextensibility may be marked or minimal. Skin splitting is limited and there is little scarring. The bruising tendency is variable in degree, but is usually not troublesome. Orthopedic complications occur in many of the patients (Beighton 1969, Beighton and Horan 1969).

Type IV.

This is the gravest form of Ehlers-Danlos syndrome. The skin is not exceptionally distensible, but it is strikingly thin and tends to be tight over the face, ears and fingers. The skin often ruptures and keloids are often formed. The subcutaneous veins are very prominent, also over the trunk. There is no impressive joint hypermobility, on the contrary, contractures are sometimes seen. The connective tissue is fragile and spontaneous ruptures of arteries and bowels are a particular feature. The ready bruising ability is very prominent (Beighton 1969, Mc Kusick 1972, Pope et al. 1975).

Type V.

In this entity joint hypermobility is only mild, while skin hyperextensibility is very prominent. The bruisability varies. Skin splitting and scarring are moderate and spheroids and molluscoid pseudotumours are seen. Orthopedic complications are common and the tissue may be friable at operation (Beighton 1969, Mc Kusick 1972, Di Ferrante et al. 1975, Siegel et al. 1979).

Type VI.

This type is characterized by kyphoscoliosis present already at birth. The scoliosis can progress during growth to considerable magnitude. The patient has recurrent joint dislocation and hyperextensible skin and joints. The scars are not abnormal in appearance. Skin bruises are common. Rupture of the sclera or cornea and detachment of the retina are seen. When skin ruptures, the edges retract with only little bleeding, as in type I. Wounds tend to heal slowly, and sutures are often left in place for an extra week. Surgical correction of the kyphoscoliosis has been performed without any complications (Mc Kusick 1972, Pinell et al. 1972).

Type VII.

This type, previously called arthrochalasia multiplex congenita, is recognized by short stature, severe joint laxity with congenital dislocations. The skin is soft and velvety. The bruisability is moderate (Mc Kusick 1972, Lichtenstein et al. 1973).

The biochemical defects.

The molecular defect in the dominantly inherited types I, II and III has not been identified but might perhaps be at the transcriptional level (Siegel et al. 1979). Type III collagen is missing in patients with EDS type IV and is thus believed to explain many of the clinical features of this disorder (rupture of arteries and intestine, thin skin with reduced extensibility, tendency to contractures of joints). In type V, the sex-linked type, a deficiency of lysyloxidase has been claimed (Di Ferrante et al. 1975). However, others have found a normal amount of the enzyme in EDS type V as well as normal proportions of cross-links in the tissues. Since the stress-strain relationship is parallel to control tissue the disorder is probably due not to a defect of the cross-linking mechanism, but as suggested in types I, II and III, to abnormal aggregation of the fibres into bundles (Siegel et al. 1979). Type VI is biochemically characterized by lack of lysyl hydroxylase with

considerably decreased hydroxylation of the lysyl residues of the collagen, which in turn results in defective cross-linking of the collagen (Pinell et al. 1972). In this recessively inherited disorder the heterozygotic state of the apparently healthy parents is indicated by reduced amounts of hydroxylysine in skin specimens (Krieg et al. 1979).

In type VII a defective conversion of procollagen to collagen is believed to be the biochemical basis of the clinical manifestations of this syndrome. A partial deficiency of procollagen amino-protease has been claimed to be the cause (Lichtenstein et al. 1973). However, more recent research suggests that the pro- α -chain in at least one patient had a structural defect near the amino-protease cleavage site preventing the removal of the amino-propeptide by this enzyme (Steinmann et al. 1979).

Incidence

The Ehlers-Danlos syndrome is rare. Beighton (1969) found a minimum over-all frequency of about 1:150,000 (128 in 20 millions) but pointed out that there must be a number of undiagnosed cases. Especially the milder cases can easily be overlooked. Broken down into the various types of EDS the approximate figures are as follows:

Type I	(Gravis)	1:625,000
Type II	(Mitis)	1:445,000
Type III	(Benign hypermobile)	1:1.8 million
Type IV	(Ecchymotic)	1:5 million
Type V	(X-linked)	1:2.5 million

The frequency of type VI and that of type VII cannot be determined with any accuracy since there are only very few cases known in which the biochemical defects have been verified (Krieg et al. 1979, Steinmann et al. 1979).

SCOLIOSIS

Classification

Scoliosis is defined as a lateral curvature of the spine. There are two main types; non-structural and structural. Non-structural scoliosis is visible only when the patient is

standing (postural scoliosis) it has no rotation, and the mobility of the spine is normal. One cause is difference in length of the legs. This type disappears when the tilting of the pelvis is corrected. This form of scoliosis is not progressive (Goldstein and Waugh 1973).

Structural scoliosis is of greater clinical importance. Besides the lateral deviation there is a fixed rotational component causing a dorsal prominence (hump) on the convex side of the curve. The deformity sometimes progresses during growth.

The classification of structural scoliosis most commonly used has been proposed by the terminology committee of the Scoliosis Research Society (Goldstein and Waugh 1973).

A. Idiopathic scoliosis

The majority of scoliosis (80-85 %) is idiopathic. According to the age of the patient at the time of recognition these cases are divided into three groups:

1. Infantile, under four years of age;
2. Juvenile, from four to nine years of age;
3. Adolescent, between ten years of age and skeletal maturity.

B. Congenital scoliosis

In this group the deformity is due to abnormal bone development with

1. Defective formation (e.g. hemivertebrae) or
2. Failure of segmentation (e.g. unilateral bar).

C. Neuromuscular scoliosis

Structural scoliosis can develop in growing children with

1. A neuropathic disease (e.g. polio and cerebral palsy) or
2. With a myopathic disease (e.g. muscular dystrophy).

D. Scoliosis associated with neurofibromatosis Recklinghausen

E. Scoliosis in mesenchymal disorders

1. Congenital (e.g. Marfan's syndrome, Ehlers-Danlos syndrome)
2. Acquired (e.g. rheumatoid arthritis).

F. Scoliosis caused by trauma

1. Vertebral (e.g. fracture, radiation and surgery)
2. Extravertebral (e.g. burns and thoracoplasty).

G. Scoliosis secondary to irritative phenomena (transient structural curves)
(e.g. spinal cord tumour, nerve root irritation).

H. Other structural scoliosis (e.g. metabolic and nutritional)

Etiology of idiopathic scoliosis

The causes of the scoliosis in most of the groups listed above seem apparent. However, in idiopathic scoliosis the etiology is by no means clear, but patients with idiopathic scoliosis differ from a random population in several respects.

There is a female predominance, which increases with the degree of curvature (Brooks et al. 1975). The patients are often tall and lean, and there is an increased rate of body growth the year before the diagnosis is made (Willner 1974). Increased serum concentrations of growth hormone and of somatomedin A have also been reported (Willner et al. 1976, Skogland and Miller 1979), a finding that could not be confirmed by other (Misol et al. 1971, Spencer 1976). Patients with adolescent idiopathic scoliosis also have a poorer postural control and a higher frequency of electronystagmographic abnormalities (Sahlstrand 1977). The frequency of EEG changes is also higher (Sahlstrand 1977). From a biomechanical point of view, the lateral curvature tends to be worse if the spine is abnormally slender or has an abnormally high deformability or is exposed to abnormal forces and movements (Schultz 1979). Thus, constitutional, hormonal, biomechanical and neuromuscular factors may have an modifying effect on the progress of the scoliosis. This multifactoral cause of idiopathic scoliosis has also been stressed by Nachemson (1979).

Genetic aspects of idiopathic scoliosis have also been thoroughly studied. There is a definite familial tendency, but the exact mode of inheritance is not known. Cowell et al. (1972) reported a radiographic study of relatives of 110 consecutive patients with idiopathic scoliosis. There were 22 probands who had no relatives with scoliosis. Among the relatives of the remaining 88, 29 % of the brothers and 43 % of the sisters had structural scoliosis.

of 10^0 or more. There was only one male to male transmission. The authors concluded that idiopathic scoliosis is inherited in a sex-linked dominant mode with incomplete penetrance. However, others (Wynne-Davies 1968, Robin and Cohen 1975) argue for an autosomal dominant or polygenic inheritance with environmental "trigger" factors at different ages. A strong support for the hypothesis of multifactorial heredity of idiopathic scoliosis is the recurrence rate in first, second and third degree relatives documented in studies by Wynne-Davies (1968), Filho and Thompson (1971) and Riseborough and Wynne-Davies (1973). The recurrence rates in first, second and third degree relatives in the three studies were 11.1 %, 2.4 % and 1.4 %; 6.6 %, 1.6 % and 1.0 %; and 7.0 %, 3.7 % and 1.6 %, respectively. The sharp drop in the incidence of scoliosis from first degree to third degree relatives is compatible with the threshold model for multifactorial inheritance proposed by Falconer (1965).

Collagen and idiopathic scoliosis

The role of collagen in idiopathic scoliosis has long received attention. The turn-over of collagen, as judged from the total excretion of hydroxyproline in urine, is increased in idiopathic scoliosis throughout adolescence (Zorab et al. 1971). But the range of variation of the values is wide and there is substantial overlapping with the control values.

Francis et al. (1977) tested collagen from skin specimens of 15 patients with adolescent idiopathic scoliosis. The extracted collagen was polymerised and its resistance against depolymerisation by NaOH was decreased in most patients with idiopathic scoliosis compared with that in 31 non-scoliotic controls. However, two of the patients with a mature skeleton had collagen of normal stability. The authors felt that a decrease in the stability of polymeric collagen reflects a similar decrease in vivo of the number or types of collagen cross-links responsible for the tensile strength of those fibres. They concluded that cross-link formation and maturation may be abnormal in idiopathic scoliosis and that this abnormality is more severe in the younger patients. They suggested that rapid growth, which leads to an increased amount of reducible cross-links combined with temporarily unstable collagen, helps to explain why scoliosis is more common in girls than in boys, in whom the adolescent growth spurt is about two years later.

The degree of curvature and mobility of the discs has been reported to vary with the total collagen content of the discs involved (Taylor et al. 1976).

To distinguish between primary and secondary changes in the connective tissue the authors examined the discs of a 15-year-old female with scoliosis and those in a normal female control, aged 16 (Bushell et al. 1978). The discs were obtained at necropsy and dissected into 13 zones from side to side. The soluble proteoglycan was removed and the insoluble residues were then digested with pepsin. The amount of collagen extracted from each zone was expressed as a percentage of the original collagen content of that zone, and the amounts obtained from four normal and four scoliotic discs were determined. The amount of collagen extracted varied inversely with the total collagen content of the normal discs, but no such inverse relationship was found in the scoliotic discs. The percentage of collagen extracted from discs of the scoliotic patient was decreased in nucleus pulposus and in the two zones close to it, compared with that in the normal discs. In the discs from the patient with scoliosis there was no difference between the concave and the convex side and the abnormality of the discs in the scoliotic girl could thus not be ascribed to mechanical loading. The results reflect qualitative differences in collagen between discs from the scoliotic girl and the controls, differences which might be interpreted in more than one way. Two possibilities are considered by the authors. The findings may be due to a predominance of type I collagen in scoliosis since pepsin is reported to extract only type II collagen from the intervertebral discs (Osebold and Pedrini 1976, Eyre and Muir 1977). The second possibility is differences in the localization of the cross-links, since pepsin at 4°C cleaves collagen only in the non-helical region (Miller 1972). It is suggested that the results might point to a central factor in the pathogenesis of scoliosis. For example, a cross-linking defect might render disc tissue less resistant to deformation once a curvature has been started by other factors. This has also been further discussed in a recent paper (Bushell et al. 1979).

The biomechanical properties of tendons and inter-spinous ligaments in idiopathic scoliosis have been studied by Nordwall (1973), who tested elastic stiffness, plastic deformation and tensile strength. He found no differences between 40 patients with idiopathic scoliosis and 20 with paralytic or congenital scoliosis. However, the elastic stiffness of the tendons was significantly correlated with the operative correction of curvature. He also studied the shrinkage due to heat denaturation as well as the collagen content of the tendons and ligaments without finding any differences between the two groups.

Bradford et al. (1977) have studied skin fibroblasts in culture. Ten patients with idiopathic scoliosis were compared with 7 age-matched controls. There was no significant difference in any parameter studied; cell layer, extractability with salt or acetic acid and the ratio between hydroxyproline to proline, except that the fibroblasts from the scoliotic patients had a lower hydroxyproline to proline ratio in the non-dialyzable media. The authors concluded that idiopathic scoliosis is not a generalized connective tissue disorder.

THE AIM OF THIS INVESTIGATION

As in other disorders with defective collagen, scoliosis is relatively common in Ehlers-Danlos syndrome in which ready bruisability is a cardinal feature. Once it had been noticed that the bleeding time in patients operated on for idiopathic scoliosis was often prolonged it was thought that both the scoliosis and the prolonged bleeding time might have something to do with a defect of the collagen.

Testing of this possibility required a method for measuring the platelet aggregating power of collagen but no suitable method was available. Interest was therefore focused on development of a method in which a very small biopsy specimen of fascia would be sufficient and in which the preparation of the collagen suspension would be so simple that the method could be used by routine in clinical practice. It was also desired to express the platelet aggregating potency of the collagen in numerical values allowing statistical treatment.

The purpose of the study was further to ascertain whether any correlation existed between the platelet aggregating power of collagen and the bleeding time and to elucidate the role played by collagen in Ehlers-Danlos syndrome and in idiopathic and other types of scoliosis.

METHODS

Collagen-induced platelet aggregation

A biopsy specimen of a muscle fascia was obtained under local anesthesia or at some operation. The specimen was stored air-tight in parafilm at -60° C. It was afterwards scraped clean in ice-cold distilled water with a blunt knife. The distilled water was repeatedly changed and the scraping was continued until the piece of tissue was free from visible muscle and fat and the water remained clear despite continued scraping (about 15 minutes). The tissue had by then split up into threads. It was freeze-dried in vacuum and cut into small pieces. 8.35 mM HAc was added in an amount equal to 50 times the weight of the specimen and homogenized for 60-90 seconds with a pestle and a glass mortar. An equal volume of distilled water was added and homogenization was repeated. During the

homogenization the mortar was held in ice-cold water. When necessary the suspension was diluted further with 1.67 mM HAc. The suspension tolerated storage for some weeks at -60°C . Before being used for aggregation of platelets the suspension was thawed and rehomogenized for 10 seconds, after which it was centrifuged at 1200 g for 30 minutes at $+4^{\circ}\text{C}$. The supernatant was removed and stored at $+4^{\circ}\text{C}$. It was used the same day for aggregation tests. Two millilitres was set aside and stored at -30°C for later determination of hydroxyproline.

Human venous blood was obtained from apparently healthy volunteers. It was allowed to flow into siliconized glass tubes containing 3.8 per cent trisodium citrate (proportion 9:1). It was centrifuged at 250 g for 10 minutes at $+4^{\circ}\text{C}$. The platelet-rich plasma was pipetted off, and the platelet count was determined (Björkman 1959). Platelet-poor plasma (PPP) was obtained by centrifugation of the residue at 1200 g for 15 minutes. The platelet-rich plasma was diluted with PPP to a platelet count of about $300 \times 10^9/\text{l}$ (PRP). Because of initial variation in responsiveness the PRP cannot be used within the first 15 minutes.

Platelet aggregation in PRP in the presence of a suspension of collagen was measured in Born's aggregometer (Born 1962). Mills and Roberts' modification of the original method was used (Mills and Roberts 1967). The modification comprises constant magnetic stirring and continuous automatic recording of transmission by a 400 M Vitatron writer. The aggregation curves were recorded at a chart speed of 4 cm/minute and the transmission was set at just above 0 per cent for PRP and at 100 per cent for PPP. The aggregation induced by collagen was taken as the maximum rate of aggregation and was calculated by drawing a tangent through the steepest part of the curve and was expressed as delta per cent transmission/minute.

In each set of experiments 0.9 ml PRP was used, to which was added 0.1 ml suspension of collagen in different concentrations.

We also used a more modern aggregometer (Payton Dual Channel Aggregation Module) which allows simultaneous testing in PRP from different persons and/or collagen from different sources. In this apparatus we used 0.45 ml PRP, to which we added 50 μl of a collagen suspension.

The aggregating capacity of a suspension of collagen was calculated as follows. The maximal slope (delta per cent transmission/minute) was plotted on a linear scale against the collagen concentration of the various dilutions on a logarithmic scale (Figure 1).

Irrespective of the collagen tested the pattern was the same, viz in one interval there was a linear correlation between the concentration of the collagen and the maximal slope (A - B in Figure 1) and this line was parallel to the corresponding line for each collagen suspension tested in the same PRP.

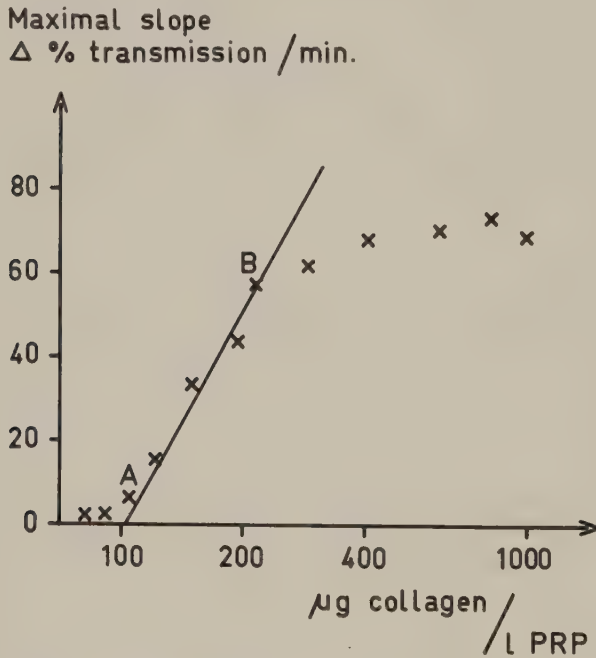


Figure 1. Variation of maximal slope in a relation to collagen concentration in PRP.

In an interval A-B the correlation is rectilinear. Critical concentration (CC) is the concentration where the A-B line crosses the X-axis. In this case CC = 100 ug collagen/l PRP.

The point of intersection between A-B and X-axis in the linear-log plot was called the critical concentration (CC) and denoted the minimal concentration of collagen in the test system that induced a release of ADP sufficient to initiate platelet aggregation. When the aggregation test was performed the hydroxyproline content of the original collagen suspension was still unknown, for which reason it was arbitrarily said to be 10 mg collagen/l. This gave a provisional CC value which was called the "10 mg/l value". To calculate the true CC the "10 mg/l value" was multiplied by the true collagen concentration determined at later measurement of the hydroxyproline and dividing the product by 10.

Since the responsiveness of PRP varies (Paper 1), the CC was also expressed in relation to a standard collagen (CC per cent) which was prepared from a fascia obtained at necropsy and stored at -30°C .

Bleeding time

The bleeding time was determined by the method of Ivy, as modified by Borchgrevink and Waaler (1958) and by Nilsson et al. (1963). A blood pressure cuff was wrapped around the upper arm and inflated to 40 mm Hg. In an area free from visible veins just below of the cubital fold, two transverse incisions were made. The incisions were made 1 mm deep either with the blade of a surgical knife (Swan Morton No. 11) or with a disposable Simplate IITM. The incisions were 10 mm long when made with a surgical blade and 5 mm when made with a Simplate IITM. The blood was blotted every 30 seconds as long as bleeding continued. The mean of the bleeding times after the two incisions was taken as the bleeding time. The reference values for both methods were the same, 6-12 minutes.

In 255 patients the surgical blade technique gave a bleeding time of 7.75 ± 2.26 (AV $\pm$ SD) and the Simplate IITM technique 7.69 ± 2.40 (AV $\pm$ SD) (Nilsson et al. 1979).

Hydroxyproline

Samples for assay were hydrolysed in 6 M HCl at 130°C for 3 hours, and hydroxyproline was determined as described by Pikkariainen (1968).

Hemostatic tests

Coagulation time in glass- and plastic-tubes, recalcification time, prothrombin consumption, bleeding time according to Duke, platelet count, platelet adhesiveness in whole blood according to the method of Hellem and according to the method of Salzman, AHF (factor VIII), haemophilia B-factor (factor IX), haemophilia C-factor (factor XI), Hageman factor (factor XII), one-stage prothrombin time, Owren's P & P (prothrombin + factor VII + factor X), factor V, fibrinogen, fibrinolytic activity of plasma and resuspended euglobulin precipitate, on unheated fibrin plates, euglobulin clot lysis time, plasminogen by an immunological method and fibrin degradation products (FDP) (Nilsson 1973).

Joint laxity

Joint laxity was assessed according to Carter and Wilkinson (1964).

Measuring of the scoliosis

The curvatures were measured with the method of Cobb (1948).

SUMMARY OF PAPERS

Paper I. A method for measuring collagen induced platelet aggregation

The effect of collagen on platelets can be studied with a photometric method described by Born (1962). In most studies interest has been focused mainly on the reaction of the platelets, but to some extent also on the effect of various preparations or different types of collagen (Wilner et al. 1968, Balleisen et al. 1975, Barnes et al. 1976, Muggli 1978). In the evaluation of the platelet aggregating power of a collagen preparation, curves obtained for equal concentrations of collagen have usually been compared to assess the relative potency of a given collagen suspension. This paper concerns a method for measuring the platelet aggregating effect of collagen and the effect of some parameters on the results. A rectilinear correlation between the response (the speed of platelet aggregation) and the logarithm of the collagen concentration made it possible to calculate a numerical value for the platelet aggregating power, a value that could be used in statistical analyses.

Collagen in solution does not aggregate platelets at all; it must repolymerize to be effective. We prepared a suspension of collagen directly by homogenising mechanically cleaned and freeze-dried fascia in 8.35 mM acetic acid. Compared with collagen suspensions made from collagen in solution, this suspension proved to be much more potent and had thus probably retained more of the original potency of the native collagen.

It was also found that the platelet aggregating power varied with the anatomic structure from which the biopsy specimen had been obtained.

Collagen in solutions and suspensions may polymerize spontaneously, and since the degree of dispersion affects the platelet aggregating power (Muggli 1978) we recommended that inter-patient comparison should be based on fascia biopsy specimens of identical anatomical structure, obtained at the same time, and stored and prepared together. Owing to a considerable inter- and intra-individual variation of the PRP the comparison of different collagen preparations is also safer if the test is performed in the same batch of platelet-rich plasma (PRP). To eliminate this variation the platelet aggregating power of a collagen suspension can also be expressed relative to a standard collagen prepared from large pieces of fascia obtained at necropsy.

Summing up, several factors can affect this in vitro determination, but an adequate standardization of the method can reduce the coefficient of variation to acceptable level (14%).

Paper II. Correlation between the bleeding time and the platelet aggregating activity of collagen in scoliosis

In 26 patients with scoliosis a significant correlation ($r = 0.40$) was found between the platelet aggregating potency of collagen and the Ivy bleeding time. This indicates that in this in vitro determination the collagen retains at least some of its native properties.

Paper III. Collagen and bleeding diathesis in Ehlers-Danlos syndrome

The Ehlers-Danlos syndrome (EDS) is characterized by unduly extensible skin with cigarette paper thin scars, friability of the connective tissue and a bleeding diathesis. There are at least seven variants of the syndrome, and a brief summary of the types are given according to their mode of inheritance, basic defects of the collagen and clinical features. Seven patients of three families are described. On the basis of the clinical findings the patients of one family were referred to type I (the gravis type); the second family, to type III (the benign hypermobile type); and the third family had only one affected member and she had an unclassifiable type of the syndrome. All the patients had bleeding symptoms, but surgery had been performed on all of them without complications due to increased bleeding or tissue friability.

The bleeding diathesis in EDS is not properly understood. Since the collagen in Ehlers-Danlos syndrome is believed to be defective and since the collagen plays an important role in hemostasis, it was thought that the collagen might be one cause of the bleeding

diathesis. However, the platelet aggregating power of the collagen was normal in our cases of EDS. An extensive examination of coagulation factors, platelet functions and fibrinolysis revealed a decreased platelet adhesiveness in only two twin boys, which might be coincidental. However, the mean bleeding time in the group of patients with EDS proved to be longer than in controls, when determined with a sensitive modification of Ivy's method.

Ehlers-Danlos syndrome is very heterogenous and surgery in such cases is often attended by complications. Experience of earlier operation on the patient as well as on affected relatives is important for detecting patients at risk. Knowledge of the type of the syndrome the patient has is essential. Preoperative examination for coincidental coagulation and bleeding disorders is recommended since many defects can be substituted or treated in other ways during surgery. There is also some evidence that this modification of Ivy's bleeding time will be sensitive enough to be useful in the preoperative evaluation of the risks. If the bleeding time proves prolonged, it would at least be of theoretical interest to estimate the platelet aggregating power of the collagen.

Paper IV. Collagen-induced platelet aggregation and bleeding time in adolescent idiopathic scoliosis

Scoliosis is common in animals and humans with defect collagen. Collagen abnormalities have been described also in idiopathic scoliosis. In this study the platelet aggregating potency of the collagen from 15 patients with idiopathic scoliosis proved to be decreased by 75 per cent, compared with that in 10 non-scoliotic controls. This alteration of the collagen may also explain the prolonged bleeding time in 68 patients with idiopathic scoliosis. The collagen concentration of the suspension prepared from fascia specimens was also higher in idiopathic scoliosis. Our observations lend support to the hypothesis that collagen is involved in the development of idiopathic scoliosis. A possible explanation of these findings is an altered cross-linking of the collagen analogous to Ehlers-Danlos syndrome type VI, which is characterized by scoliosis and a bleeding tendency and in which the basic defect is a decreased cross-linking due to lack of collagenlysin hydroxylase. Another possibility is a decreased content of collagen type III, which has an increased platelet aggregating power or, thirdly, an otherwise altered structure of the collagen.

Paper V. Collagen changes in congenital and idiopathic scoliosis

Two out of four cases with congenital scoliosis showed the same abnormalities of the collagen as in idiopathic scoliosis i. e. a decrease of the platelet aggregating potency and an increased amount of collagen in suspensions prepared from fascia. Since the biopsy specimens were obtained at operations of patients with scoliosis and since only relatively few cases with congenital and idiopathic scoliosis deteriorate to such a degree as to require surgery, it was thought that this collagen abnormality might render scoliosis more liable to progress.

In idiopathic as well as in congenital scoliosis the decrease in the platelet aggregating power was more conspicuous in younger patients. This was also the case with the alteration of the collagen described by Francis et al. (1977).

Paper VI. Bleeding time in scoliosis

Since there is a correlation between the platelet aggregating power of the collagen and the bleeding time in patients with scoliosis and since the determination of the bleeding time is a relatively simple procedure, the bleeding time was determined also in patients with small scoliosis from whom biopsies could not be obtained. A sensitive modification of Ivy's method was used. The mean value of the bleeding time was higher in females, especially in females with idiopathic scoliosis. Patients with paralytic scoliosis had a significantly longer bleeding time than non-scoliotic controls. The patients with congenital scoliosis did not differ significantly from the controls or the other types of scoliosis. There was no correlation between age and bleeding time. Since there was no correlation between the bleeding time and the magnitude of the scoliosis this collagen abnormality will presumably not have any major influence on the progress of the curvature in contradiction of that proposed in paper V. This lack of correlation also argues against this abnormality being secondary to the scoliosis for otherwise the abnormality should be more pronounced in greater curves.

DISCUSSION

In our investigation we used a modification of Born's method in our study of collagen induced platelet aggregation. The modification consisted essentially of a difference in the evaluation of the curves. We used the rate of platelet aggregation because in a part of the dose response curve there is a rectilinear correlation between it and the logarithm of collagen concentration in PRP. The intersection of the extension of this line and the X-axis in a line-log plot made it possible to define a point corresponding to a concentration of collagen in PRP just above that capable of inducing platelet aggregation. This concentration is easier to reproduce than the lowest concentration owing to the sigmoid shape of the dose response curve. This rectilinear correlation has been known for some years (Muggli and Baumgartner 1973, Heyns et al. 1974, Muggli 1978) but as far as we know it has never been utilized to achieve a numerical value that can be used in statistical analyses.

The way in which the collagen suspension is prepared is crucial. Solutions of collagen do not aggregate platelets at all (Muggli and Baumgartner 1973) and the way in which the collagen is repolymerized has a strong effect on the platelet aggregating properties (Muggli and Baumgartner 1973, Simons et al. 1975). We used crude suspensions prepared directly from freeze-dried fascia. The advantage of this is that the procedure is simple and requires only a small biopsy specimen. The platelet aggregating potency is also substantially greater than that of collagen suspensions prepared from well defined collagen solutions. The critical concentration of collagen in our test system is equal to the active portion of collagen in suspensions prepared from soluble collagen (Kronick and Jimenez 1979). Directly prepared collagen suspensions probably contain also other substances, such as glucose amino glucans, which can interfere with platelet aggregation, but the differences in solubility of the various types of collagen is assumably of less importance in our method. Since the platelet aggregating potency is considerably greater, the collagen of this preparation probably contains more of the original properties of the native collagen and we believe its advantages to outweigh its disadvantages, an assumption supported also by the correlation between the bleeding time and the platelet aggregating power of the collagen described in paper II.

Variation of the PRP is an important source of error in the evaluation of the platelet aggregating properties of collagen, but this can be overcome by the use of a standard collagen as reference and of a factor for correction of the age of PRP.

There are thus several factors capable of influencing this in vitro determination of the platelet aggregating potency of collagen, but standardization of the various steps reduces the coefficient of variation to 14 per cent, which can be regarded as an acceptable level for a biologic method.

The bleeding time may be prolonged in von Willebrand's disease (Nilsson et al. 1957) and in patients with defective platelets (Caen 1972) or defective collagen. (Paper VI). The coefficient of correlation between the platelet aggregating potency of the collagen and the bleeding time of Ivy was found to be 0.40, which is comparable to the correlation coefficient (0.47) between the bleeding time (Ivy) and the platelet adhesiveness (Salzman 1963). But the correlation between the bleeding time (Ivy) and factor VIII in von Willebrand's disease was somewhat higher (0.67) (Silwer 1973).

It must, however, be emphasized that the prolongation of the bleeding time in scoliosis is slight, and such a prolongation should not be expected to produce any bleeding symptoms (Nilsson et al. 1963, Silwer 1973). This alteration of the collagen, thus, has no apparent clinical significance in the hemostatic process.

Several investigations have been made to find the cause of the bleeding diathesis in Ehlers-Danlos syndrome but no consistent abnormality has been found (McKusick 1972).

The nature and role of the collagen abnormalities in the different types of Ehlers-Danlos syndrome are by no means properly understood. In type IV, where the bleeding symptoms are most severe, the patients lack type III collagen which, at least in vitro, is the most potent inducer of platelet aggregation (Balleisen et al. 1975). It is therefore tempting to ascribe the bleeding diathesis in this variant to a decreased platelet aggregating potency of the connective tissue in the vessel walls. However, since also type III collagen is suited to work in distensible tissues, another explanation may be friability of the vessels. This view is also supported by the frequently positive tourniquet test (Frick and Krafchuck 1956, Barabas and Barabas 1967, Hathaway 1971).

Paper III concerns 7 relatively mild cases of EDS in which the platelet aggregating properties of the collagen proved normal. The bleeding time was slightly prolonged in 3 out of 7 cases and the mean value of the group was statistically significantly prolonged. Since this modification of Ivy's method is sensitive enough to detect even minor defects in the hemostatic system (Nilsson et al. 1963) and since the bleeding time was considerably prolonged in a case of Ehlers-Danlos syndrome with severe bleeding symptoms (Karaca et al. 1972) this method is believed to be of value in the evaluation of the per-operative risks of patients with EDS.

The normal bleeding time in EDS reported by other authors may be explained by the use of less sensitive methods (Frick and Krafchuck 1956, Barabas and Barabas 1967, Hathaway 1971). For instance, the bleeding time (Duke) was normal despite severe bleeding symptom in a case of EDS, where the bleeding time (Ivy) was > 30 minutes (Karaca et al. 1972).

Besides determination of the bleeding time a preoperativa examination for coincidental coagulation or bleeding disorders is recommended, since several abnormalities have been reported in conjunction with EDS (McKusick 1972) and these can be substituted or treated in association with surgery. However, experience of earlier operations on such patients as well as on affected relatives is important for detecting patients at risk since the clinical features are relatively constant within the same family. If no such information is available knowledge of the variant to which a given case belongs may be helpful. Type IV patients and some patients of type I are high risk patients (Beighton 1969).

The aim of papers IV, V and VI was to elucidate the role of collagen in the etiology of idiopathic scoliosis. There are reports on abnormalities of the collagen in idiopathic scoliosis, but we know very little about their nature owing to our lack of knowledge of mature collagen in normal and abnormal connective tissue. For instance, the nature of the cross-links is not known. Nor is the higher organization of collagen in the tissues known, and the way different types of collagen act together to give different types of connective tissue their different properties still challenges research.

Available data will hardly allow more than speculation on the nature of the collagen abnormalities in idiopathic scoliosis. Investigation of fibroblasts in culture has failed to reveal any major abnormality of the collagen molecule (Bradford et al. 1977). A decreased stability of extracted polymeric collagen of young patients with idiopathic scoliosis has been ascribed to abnormal cross-linking of the collagen (Francis et al. 1977). However, the biomechanical properties of tendons and ligaments in idiopathic as well as in congenital and paralytic scoliosis are normal also in young patients (Nordwall 1973). Thus, the cross-linking is probably not impaired to any great extent. A decreased content of type I collagen in the intervertebral discs has been suggested to make curvatures more prone to progress once scoliosis has been started by other factors (Bushell et al. 1978). The decreased platelet aggregating potency of the collagen described in papers IV and V may be due to a decreased proportion of type III collagen in the ligaments, which may in turn increase the liability of a curvature to progress. But there are certainly also other possible explanations. Since the collagen was defective also in fascias distant from the scoliosis and since the bleeding time was prolonged, the collagen abnormalities are probably generalized.

Abnormalities of the collagen are probably not the only cause of idiopathic scoliosis since a multifactorial etiology has been suggested by the mode of inheritance (Wynne-Davies 1968, Filho and Thompson 1971, Riseborough and Wynne-Davies 1973) and by the many reports of abnormal findings in these patients. They are asymmetric with a difference in length of the arms and they are plagiocephalic (Burwell et al. 1976). They are tall and lean and have an increased rate of growth (Willner 1974) and the insecretion of growth stimulating hormones such as growth hormone and somatomedin A (Willner et al. 1976) or androgens (Rappaport et al. 1974, Skogland and Miller 1979) is increased.

The patients have poor postural stability (Sahlstrand 1977) and the frequency of EEG abnormalities is increased (Petersén et al. 1979). The muscles show asymmetrical strength and the muscle spindles are hypersensitive (Hoogmartens and Stuyck 1978). Thus, the development of a scoliosis can be the result of several factors and a collagen defect alone may not always be enough to cause the condition since not all patients with Ehlers-Danlos syndrome develop scoliosis (Beighton and Horan 1969). However, as described in paper IV, there was a considerable difference between the collagen in idiopathic scoliosis and normal collagen with only a minimum of overlapping. Abnormalities

of the collagen may therefore be one of the more important factors.

The multifactorial approach to the problem can also explain why some of the patients with congenital scoliosis also show collagen abnormalities and why the bleeding time is prolonged also in paralytic scoliosis. (Francis et al. 1976) (Paper V). Moreover the prognosis of a congenital scoliosis does not depend only on malformation of the vertebrae (Winter et al. 1968). The multifactorial hypothesis is also supported by the inverse correlation between the degree of the curvature and the logarithm of the incidence (Kane 1977) i.e. severe scoliosis is much rarer than mild scoliosis. If there was only one causal factor of idiopathic scoliosis, there should also be a good correlation between this factor and the severity of the scoliosis, but hitherto no such correlation has been reported (Willner 1974, Francis et al. 1977, Sahlstrand 1977) (Paper VI). The data in papers IV, V and VI suggest that collagen plays a role in the development of idiopathic, congenital and paralytic scoliosis, and at least in idiopathic scoliosis the collagen seems to differ considerably from normal.

GENERAL SUMMARY AND CONCLUSIONS

A method for quantification of collagen-induced platelet aggregation is described. Native collagen in suspensions prepared by direct homogenization of freeze-dried fascia probably retains more of the original platelet aggregating properties than suspensions prepared from chemically well defined solutions of collagen. The in vitro values recorded varied with the anatomical structure from which the biopsy specimens were obtained and the platelet-rich plasma by which the test was performed. The variation was reduced to acceptable level by standardization of the various steps of the method. There is a correlation between the in vitro platelet aggregation potency of collagen and the in vivo determined bleeding-time performed according to a sensitive modification of Ivy's method.

The collagen from 7 patients with the Ehlers-Danlos syndrome had normal platelet aggregating properties and the bleeding time (Ivy) was only slightly prolonged. There were three patients from a family with type I of the syndrome (gravis type), three from a family with type III (benign and hypermobile type) and one patient with an unclassifiable type of the syndrome. Despite their bleeding tendency all had been operated on several times

without any bleeding complication.

In idiopathic scoliosis the platelet aggregating potency of the collagen was decreased and suspensions made from fascia contained an increased amount of collagen, which can be caused by an altered structure of the connective tissue. The collagen in two out of four cases of congenital scoliosis showed similar abnormalities. The alteration of the collagen was also probably reflected in a slightly prolonged bleeding time, which is of no clinical importance but which may elucidate the role of collagen in the etiology of idiopathic scoliosis which appears to be multifactorial. Collagen may play a role not only in the etiology of idiopathic scoliosis, but also in paralytic and congenital scoliosis, in which similar abnormalities were found.

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A METHOD FOR MEASURING COLLAGEN-INDUCED PLATELET AGGREGATION

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ABSTRACT

Altered collagen plays an important role in some diseases. A method is described for quantitation of collagen-induced platelet aggregation. Several factors affect this in vitro determination and a high degree of standardization is required. Comparison of collagen from different persons must rely on biopsy specimens of identical anatomical structure. The aggregation test must be performed with the same platelet-rich plasma (PRP) because of considerable inter- and intra-individual variation. Standardization reduces the variation to acceptable level (14 %).

INTRODUCTION

Contact between platelets and denuded collagen in a vessel wall initiates hemostasis. It releases ADP, which stimulates platelet aggregation. It is now known that the actual adhesion to the collagen does not require energy and that the collagen triggers off an energy-requiring release of endogenous ADP from the granules of the platelets (1). In some diseases collagen aggregates platelets in a subnormal way e.g. scurvy (2) (increased bleeding), Ehlers-Danlos syndrome (3) (ready bruisability) and in some patients with prolongation of the bleeding time as the only demonstrable disorder of the coagulation system (2). The collagen may also be hyperactive, as it is, for example, in lathyrism (4) and sometimes in patients with recurrent thrombosis (5). Collagen of type III is a very potent stimulant of platelet aggregation (6) and the composition of the collagen in arteriosclerotic vessel walls differs from that in normal vessels (7). It was therefore thought worth while to

assess the ability of such collagen to stimulate platelet aggregation.

This paper concerns a routine clinical method for estimating the ability of collagen to aggregate platelets and certain technical factors to be considered in the interpretation of the results.

MATERIAL AND METHODS

Preparation of the collagen solution

A biopsy specimen of a muscle fascia was obtained under local anesthesia or at some operation. The specimen was stored airtight in parafilm at -60°C . It was afterwards scraped clean in ice-cold distilled water with a blunt knife. The distilled water was repeatedly changed and the scraping was continued until the piece of tissue was free from visible muscle and fat and the water remained clear despite continued scraping (about 15 minutes). The tissue had by then split up into threads. It was freeze-dried in vacuum and cut into small pieces. 8.35 mM HAC was added in an amount equal to 50 times the weight of the specimen and homogenized for 60-90 seconds with a pestle and a glass mortar. An equal volume of distilled water was added and homogenization was repeated. During the homogenization the mortar was held in ice-cold water. When necessary the suspension was diluted further with 1.67 mM HAC. The suspension tolerated storage for some weeks at -60°C . Before being used for aggregation of platelets the suspension was thawed and rehomogenized for 10 seconds, after which it was centrifuged at 1200 g for 30 minutes at $+4^{\circ}\text{C}$. The supernatant was removed and stored at $+4^{\circ}\text{C}$. It was used the same day for aggregation tests. Two ml was set aside and stored at -30°C for later determination of hydroxyproline. The hydroxyproline was measured with a modification (8) of Woessner's method (9). The collagen content was calculated by multiplication of the hydroxyproline concentration by 7.1.

Preparation of platelets

Human venous blood was obtained from apparently healthy volunteers. It was allowed to flow into siliconized glass tubes containing 3.8 % trisodium citrate (proportion 9:1). It was centrifuged at 250 g for 10 minutes at $+4^{\circ}\text{C}$. The platelet-rich plasma was pipetted off and the platelet count was determined (10). Platelet-poor plasma (PPP) was obtained by centrifugation of the residue at 1200 g for 15 minutes. The platelet-rich plasma was diluted with PPP to a platelet count of about $300 \times 10^9/\text{l}$ (PRP). Because of initial variation in responsiveness the PRP cannot be used within the first 15 minutes.

Calculation of ability of collagen to aggregate platelets

Platelet aggregation in PRP in the presence of a suspension of collagen was measured in Born's aggregometer (11). Mills and Roberts' modification of the original method was used (12). The modification comprises constant magnetic stirring and continuous

automatic recording of transmission by a 400 M Vitatron writer. The aggregation curves were recorded at a chart speed of 4 cm/min and the transmission was set at just above 0 % for PRP at 100 % for PPP. The aggregation induced by collagen was taken as the maximum rate of aggregation and was calculated by drawing a tangent through the steepest part of the curve and was expressed as Δ % transmission/min (Fig. 1).

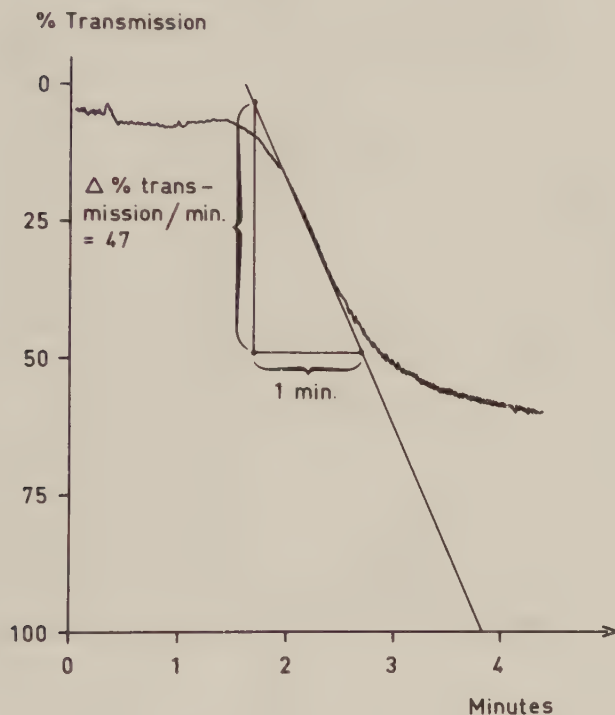


FIG. 1

Calculation of maximal slope of an aggregation curve.

In each set of experiments 0.9 ml PRP was used, to which was added 0.1 ml suspension of collagen in different concentrations.

We also used a more modern aggregometer (Payton Dual Channel Aggregation Module) which allows simultaneous testing in PRP from different persons and/or collagen from different sources. In this apparatus we used 0.45 ml PRP, to which we added 50 μ l of a collagen suspension.

The aggregating capacity of a suspension of collagen was calculated as follows. The maximal slope (Δ % transmission/min) was plotted on a linear scale against the collagen concentration of the various dilutions on a logarithmic scale (Fig. 2).

Irrespective of the collagen tested the pattern was the same; viz in one interval there was a linear correlation between the concentration of the collagen and the maximal slope (A-B in Fig. 2) and this line was parallel to the corresponding line for each collagen suspension tested in the same PRP.

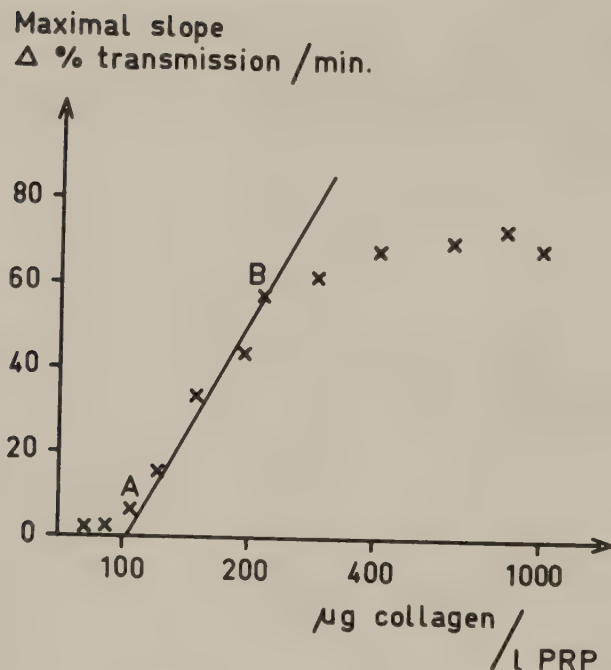


FIG. 2

Variation of maximal slope in a relation to collagen concentration in PRP. In an interval A-B the correlation is rectilinear. Critical concentration (CC) is the concentration where the A-B line crosses the X-axis. In this case $CC = 100 \mu\text{g collagen/l PRP}$.

The point of intersection between A-B and X-axis in the linear-log plot was called the critical concentration (CC) and denoted the minimal concentration of collagen in the test system that induced a release of ADP sufficient to initiate platelet aggregation. When the aggregation test was performed the hydroxyproline content of the original collagen suspension was still unknown, for which reason it was arbitrarily said to be 10 mg collagen/l . This gave a provisional CC value which was called the " 10 mg/l value". To calculate the true CC the " 10 mg/l value" was multiplied by the true collagen concentration determined at later measurement of the hydroxyproline and dividing the product by 10.

RESULTS

To assess the effect of the individual steps of the method on the reproducibility of the results the experimental conditions were varied.

Effect of age of PRP

The reactivity of the platelets varied inversely with the interval between the preparation of PRP and the test. This meant that the longer the interval the larger amount of collagen necessary for a given rate of platelet aggregation. In other words, the older the preparation the higher the CC. By determining CC for a collagen at intervals a factor for correction of age can be calculated which could be used when comparing other suspensions of collagen tested in the same PRP. Fig. 3 illustrates such an operation.

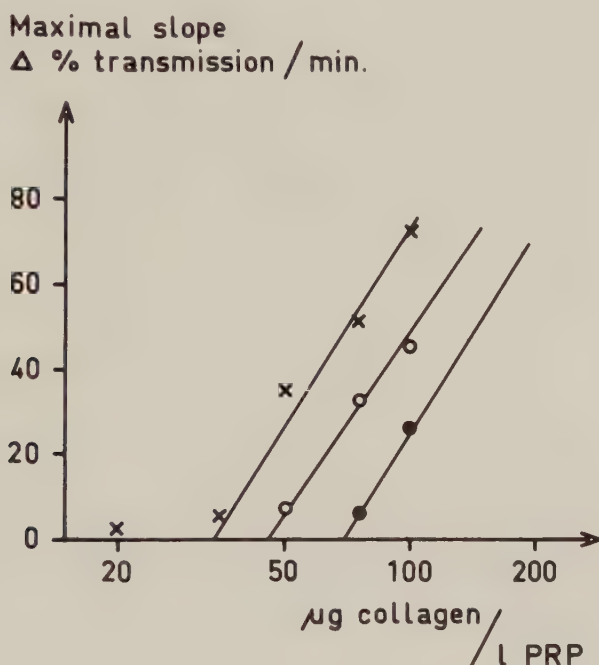


FIG. 3

Critical concentration (CC) of a collagen suspension determined in PRP of different ages. X = collagen tested in PRP 15 minutes old. o = PRP 135 minutes old and ● = PRP 195 minutes old.

$CC_{15 \text{ min}} = 34 \text{ } \mu\text{g collagen/l PRP}$, $CC_{135 \text{ min}} = 46 \text{ } \mu\text{g collagen/l PRP}$, and $CC_{195 \text{ min}} = 70 \text{ } \mu\text{g collagen/l PRP}$.

Factor for correction of age at 135 minutes = $34/46 = 0.72$ and at 195 minutes = $34/70 = 0.49$.

Effect of site of biopsy and size of specimen

Thirteen pieces of fascia were obtained from an abdominal muscle and 6 from fascia lata femoris. The dry weights of the specimens ranged from 42 to 107 mg (Table I).

The final CC value did not vary significantly with the size of the specimens ($r_{df\ 19} = -0.38$). A significant difference was, however, found between the specimens obtained from the two sites (Student's t-test, Table I).

The concentration of collagen in the original suspensions ranged from 11.5 to 114.6 mg/l. There was no significant correlation between the concentration and the final CC value ($r_{df\ 19} = -0.22$).

TABLE I
Critical concentration of collagen-induced aggregation

Biopsy from	Dry-weight	Collagen	"10 mg/l critical concentration"		Critical
fascia	mg	conc. in	uncorrected	corrected for	conc.
lata		original	value $\mu\text{g/l}$	age of PRP	$\mu\text{g colla-}$
		suspension		$\mu\text{g/l}$	gen/l PRP
		mg/l			
1	64	12.7	150	100	127
2	88	12.3	160	100	123
3	72	11.5	110	110	126
4	56	17.4	82	82	143
5	42	41.6	50	40	166
6	46	54.9	55	46	253
					$x = 156$
					$SD = 50$
					$c/v = 32\%$
Biopsy from					
muscle fascia					
1	68	31.0	18	18	56
2	100	53.4	21	20	107
3	67	56.0	15	12.5	70
4	98	33.3	30	22	73
5	69	61.3	26	24	147
6	107	52.6	33	24	126
7	103	89.0	9	7	62
8	106	44.9	41	27	121
9	92	114.6	10.5	7.5	89
10	46	78.9	11.5	10	79
11	67	93.8	13	8	75
12	78	22.7	50	36	82
13	89	20.1	95	56	113
					$x = 92$
					$SD = 28$
					$c/v = 30\%$

Student's t-test for difference between fascia lata and abdominal muscle fascia $p < 0.01$

Interindividual variation of PRP

The CC for a suspension of collagen was determined using PRP from seven apparently healthy donors. The value was found to range from 60-140 μg collagen/1 PRP (median 90). One suspension of collagen was tested on 4 occasions in PRP obtained from one and the same person at intervals of a few days. The variation in that person was roughly only one fourth of that between individuals (Table II).

TABLE II

Critical concentration of a collagen suspension in PRP from seven persons (A) and in PRP from a single person on four different days (B).

μg collagen/ <u>1</u> PRP	
A	B
60	150
75	175
80	180
90	190
90	
110	
140	

Coefficient of variation for tests performed on different days

The aggregation tests summarized in Table I were made on different days within a 3-week period. The mean CC varied somewhat from day to day, but not significantly (small groups). For the period as a whole the coefficient of variation (c/v) was about 30 % for fascia from the abdominal muscles as well as for specimens from the fascia lata. The c/v for the individual days was 18 %.

Maximal standardization

When determining the CC of 10 biopsy specimens of fascia lata we tried to keep the testing conditions as constant as possible. The aggregation tests were performed within a 2-day period and with PRP not older than 1 hour. No correction factor was necessary. The coefficient of variation was then 14 % (Table III).

TABLE III

Critical concentration of collagen capable of inducing platelet aggregation. Standardized conditions. Ten fascia specimens from a single person. No correction for age of PRP

Biopsy from fascia lata	Collagen conc. in original susp. mg/ <u>l</u>	"10 mg CC" μg/ <u>l</u>	Critical conc. μg collagen/ PRP
1	14.3	55	79
2	19.6	40	78
3	16.3	44	72
4	19.3	31	60
5	13.4	46	62
6	9.6	71	68
7	16.6	45	75
8	18.1	32	58
9	12.3	74	91
10	15.4	45	69

$x = 71$

$SD = 10.1$

$c/v = 14 \%$

DISCUSSION

Since the preparation technique interferes with the ability of collagen to induce platelet aggregation the production of the suspension or the solution of collagen must be carefully defined and comparison of collagen prepared in different ways is difficult to evaluate. For instance treatment of the experimental animal with beta-amino-propio-nitrile will increase the aggregating effect of its collagen two- to three-fold (13). The structure of collagen varies with the site of the fascia from which it is prepared. The fascia lata is more tendinous than that of the abdominal wall and the difference in its effect on platelets (Table I) may depend on differences in cross linking. There is no investigation concerning that but the cross linking in tendon differs from that in skin (14). Therefore, interpatient comparisons must be based on biopsy specimens from identical anatomical structures.

The preparation as well as the storage of collagen suspensions requires the use of a constant standard procedure. However, there may be a slight variation due to differences in the cleaning procedure, the lyophilization, the storage or accidental thawing of the samples adding to the total variation in platelet aggregation of collagen. In the comparison of subjects the samples used should be produced and stored together and tested in the same PRP. Also as an internal standard, a slice of fascia with known ability to aggregate platelet may be used.

Since vessels and other tissues may be composed of mixed types of collagen (7, 14) with varying solubility we used a collagen suspension. A suspension is easier to prepare and

retains more of its original properties with respect to the platelet aggregation. Our test model requires about 100 μ g of collagen/1 of PRP to induce platelet aggregation. In studies using solutions of collagen the corresponding concentration is about 20 times as high (4, 6, 13, 17). This implies that the dissolved collagen has lost at least 95 % of its original ability to aggregate platelets.

Interindividual variation of PRP is an important source of error in the evaluation of collagen to aggregate platelets. Ten Cate et al (15) have reported about defect collagen-induced platelet aggregation in a normal population. Between the seven healthy volunteers described above (Table III) there was a difference of about 100 % between the highest and the lowest values. Also intraindividual values can vary from one occasion to another with, among other things, the diet (16). In the determination of CC we introduced an intermediate step namely "10 mg CC". This means that the aggregation test was performed before we knew the concentration of the collagen in the suspension. This was for two reasons. First, to avoid the risk of increased variation of the polymerization of the collagen if the suspension is allowed to stand too long. Such an increase that would influence its ability to aggregate platelets (18). Second, lack of knowledge of the true concentration of collagen in the suspension and thereby also of expected values reduces the risk of bias on the part of the examiner in the determination of the CC. The line connecting the points denoting the maximal slope values is not always straight and then the examiner must himself decide where the line should be drawn. The responsiveness of PRP varies with time. Rossi and Louis (19) describe an initial increase followed by a subsequent decrease. We found that PRP was not reliable during the first 15 minutes but from that point on the correction factor can be used since there is a steady decline of responsiveness.

In conclusion, several factors affect the in vitro determination of the ability of collagen to aggregate platelets, but standardization of every step in the method can reduce the variation to an acceptable level. This method can be used in clinical practice and in research such as on the comparison vessels from patients with arteriosclerosis or diabetes as well as in the investigation of the effect of blocking agents on platelet aggregation.

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CORRELATION BETWEEN THE BLEEDING TIME AND THE PLATELET
AGGREGATING ACTIVITY OF COLLAGEN IN SCOLIOSIS

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ABSTRACT

In 26 patients with scoliosis a significant correlation was found between the platelet aggregating properties of the collagen and the Ivy bleeding time.

I N T R O D U C T I O N

Contact between platelets and denuded collagen in a vessel wall initiates hemostasis (1). An abnormal collagen with impaired ability to aggregate platelets have been described in scurvy (2), in Ehlers-Danlos syndrome (3), in idiopathic scoliosis (4) and in some patients with a prolonged bleeding time (5). In idiopathic scoliosis the bleeding time is also prolonged (4). The basic defect of the connective tissue in idiopathic scoliosis is not known. Nor is it known why the platelet aggregating power of collagen is decreased. Altered cross-linking of the collagen and decreased content of collagen of type III have been discussed (4). This paper reports a correlation between the platelet aggregating properties of collagen and the bleeding time of Ivy.

M A T E R I A L A N D M E T H O D S

Fascia specimens were obtained from the longitudinal dorsal muscles of 26 patients operated on for scoliosis. The condition was congenital in four and idiopathic in 22 patients. The curvature ranged from 50-100° according to Cobb (6). The plate-

let aggregating power of the collagen was determined as described in detail elsewhere (7). The pieces of fascia were freeze-dried in vacuum and homogenized in 8.35 mM acetic acid. After centrifugation the concentration of collagen in the supernatant was estimated from the content of hydroxyproline. The aggregating ability of the collagen was assessed photometrically. A Payton Dual Channel aggregating Module was used. The lowest concentration of collagen in platelet-rich plasma (PRP) that induced platelet aggregation was called the critical concentration (CC). PRP was obtained from healthy volunteers. Since the responsiveness of PRP varies, the concentration of collagen necessary for platelet aggregation was expressed relative to that of a standard collagen (CC %). The bleeding time was determined according to Ivy (8), before the operation. ASA was routinely discontinued a week before the test. Since there is a semi-logarithmic relationship between concentration of collagen in PRP and speed of platelet aggregation we used the logarithm of CC % in the Pearson correlation test.

RESULTS

The correlation coefficient (r_{df24}) between the bleeding time (Ivy) and the logarithm of the relative critical concentration of collagen was 0.40. This was found to be statistically significant ($p < 0.05$) (see Fig. 1).

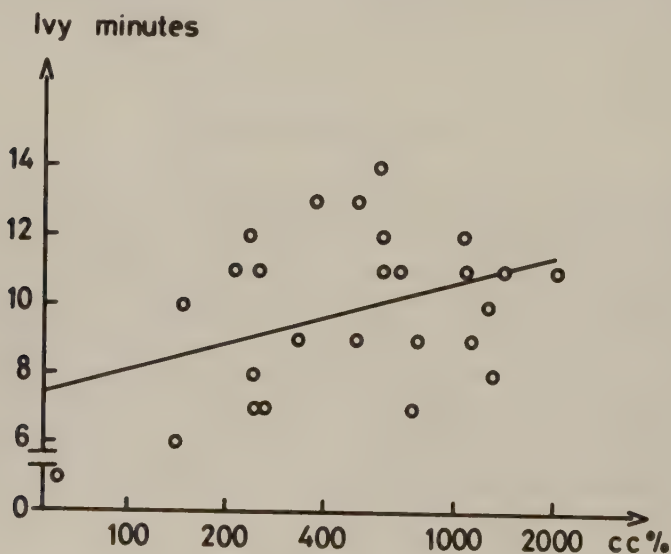


FIG. 1

The relation between the bleeding time of Ivy and platelet aggregating properties of collagen in patients with scoliosis. The concentration of collagen in PRP necessary for platelet aggregation is called critical concentration (CC). CC % means that CC is expressed as % of a standard collagen. The line of regression is calculated from $y = 1.1 \log x + 3.1$. Where y = bleeding time according to Ivy in minutes and x = CC %.

DISCUSSION

A prolonged bleeding time is seen in patients with defective platelets such as in thrombasthenia (9) and in patients with defect factor VIII as in von Willebrand's disease (10). But also defective collagen can prolong the bleeding time, as mentioned in the Introduction. However, this is the first report, as far as we know, of a statistically significant correlation between the properties of the collagen and the bleeding time. The correlation is not strong, which is only natural, since also other factors, such as platelets and the von Willebrand factor have an important influence on the bleeding time.

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PAPER III

COLLAGEN AND BLEEDING DIATHESIS IN EHLERS-DANLOS SYNDROME

ABSTRACT

There are at least seven variants of Ehlers-Danlos syndrome (EDS). One family with type I, one with type III and one isolated case with an undefined type of EDS are described. They all had bleeding symptoms, but surgery had been performed without complications. Collagen is believed to be defective in EDS. However, in our cases the platelet aggregating power of the collagen was normal. The bleeding time (Ivy) was slightly prolonged. Extensive examination of the haemostatic process revealed a decreased platelet adhesiveness in only two of the patients, a finding believed to be simply coincidental. The history of former operations on the patients or on affected relatives is considered to be the best guide when determining the risk of an operation, but preoperative estimation of the bleeding time according to a modification of the Ivy method is recommended.

INTRODUCTION

The Ehlers-Danlos syndrome (EDS) is characterized by unduly extensible skin with typical cigarette-paper thin scars, hypermobile joints, fragility of the connective tissue and a bleeding diathesis (Beighton 1969, McKusick 1972). The joint instability and the fragility of the tissues in this rare familial disorder often create orthopaedic problems (Beighton and Horan 1969a). At least seven types of the syndrome have been accepted (Beighton 1969, McKusick 1972). Synonyms, mode of inheritance, basic defects and clinical features are summarized in Table III:1. Distinction between types I, II and III is based on clinical features and there is a certain variation within each of the types (Beighton 1969). It is therefore not sufficient to assign a case to a certain type without description of the clinical features.

Table III:1. Variants of Ehlers-Danlos syndrome.

<u>Type</u>	<u>Name</u>	<u>Mode of inheritance</u>	<u>Basic defect</u>	<u>Clinical features</u>
I	Gravis type	Autosomal dominant	Lack of integrity of the fibre bundles (Siegel et al. 1979)	Classic fractures, all severe, see text
II	Mitis type	Autosomal dominant	Lack of integrity of the fibre bundles (Siegel et al. 1979)	Classic fractures, all mild, see text
III	Benign hypermobile type	Autosomal dominant	Lack of integrity of the fibre bundles (Siegel et al. 1979)	Generalized marked joint hypermobility without skeletal deformity, minimal skin features
IV	Echymotic, arterial or Sach's type	Autosomal recessive	Lack of type III collagen (Pope et al. 1975)	Very thin skin tight over the face, prominent veins, rupture of large arteries, minimal joint laxity. Severe bruisability
V	X-linked type	X-linked recessive	Lack of lysyloxidase (Di Ferrante et al. 1975). Lack of integrity of the fibre bundles (Siegel et al. 1979)	Marked skin hyperextensibility. Scarring and skin splitting moderate. Minimal joint hypermobility. Tissues may be friable
VI	Ocular type	Autosomal recessive	Lack of collagen lysylhydroxylase (Pinnell et al. 1972)	Kyphoscoliosis at birth, recurrent joint dislocation, retinal detachment, ocular rupture, moderate skin features
VII	Arthrochalasia multiplex congenita	Autosomal recessive	Defective conversion of procollagen to collagen (Steinman et al. 1979)	Short stature. Severe joint laxity with congenital dislocations. Moderate skin extensibility and bruisability

Collagen, the most abundant macromolecule of connective tissue, is believed to be defective in Ehlers-Danlos syndrome. Types IV, V, VI and VII have chemically defined abnormalities (Table III:1) (Pinell et al. 1972, Lichtenstein et al. 1973, Pope et al. 1975, Di Ferrante et al. 1975). The enzyme defect in type V has, however, been questioned (Siegel et al. 1979).

Collagen is not only responsible for the tensile strength of connective tissue, for it also plays an important role in normal hemostasis. Damage to the endothelial lining with exposure of the subendothelial collagen, to which platelets adhere, results in a series of reactions leading to the formation of a platelet thrombus (Gordin 1976).

This study concerns the platelet aggregating properties of collagen, the bleeding diathesis and its importance in surgery of patients with Ehlers-Danlos syndrome.

METHODS

The patients were examined clinically to distinguish the type of EDS. The joints were tested (Figure III:1) according to Carter and Wilkinson (1964). Notes were made of the tissue fragility and bleeding complications, if any, at previous operations. The author had operated on all but one of the patients. In conjunction with an operation fascia specimens were obtained for testing the platelet aggregating power of the collagen. Control fascia was obtained from other patients subjected to a similar operation. The collagen of the fascia was tested as described by Udén and Nilsson (1978). The pieces of fascia were scraped clean, freeze-dried in vacuum, homogenized in ice-cold 8.35 mM acetic acid and centrifuged at 1200 g for 30 minutes. The amount of collagen in the supernatant was calculated from the hydroxyproline content. The platelet aggregating power of the collagen was assessed photometrically. A Payton Dual Channel Aggregation Module was used. It includes constant magnetic stirring and constant recording of the transmission. The lowest concentration of collagen in platelet-rich plasma (PRP) that induced platelet aggregation was called the critical concentration (CC). PRP was obtained from healthy volunteers. Since the responsiveness of PRP, even from healthy persons, varies, the CC of the collagen from patients and from controls were expressed relative to that of a standard collagen (CC%), prepared from a slice of fascia with known platelet aggregating

power.

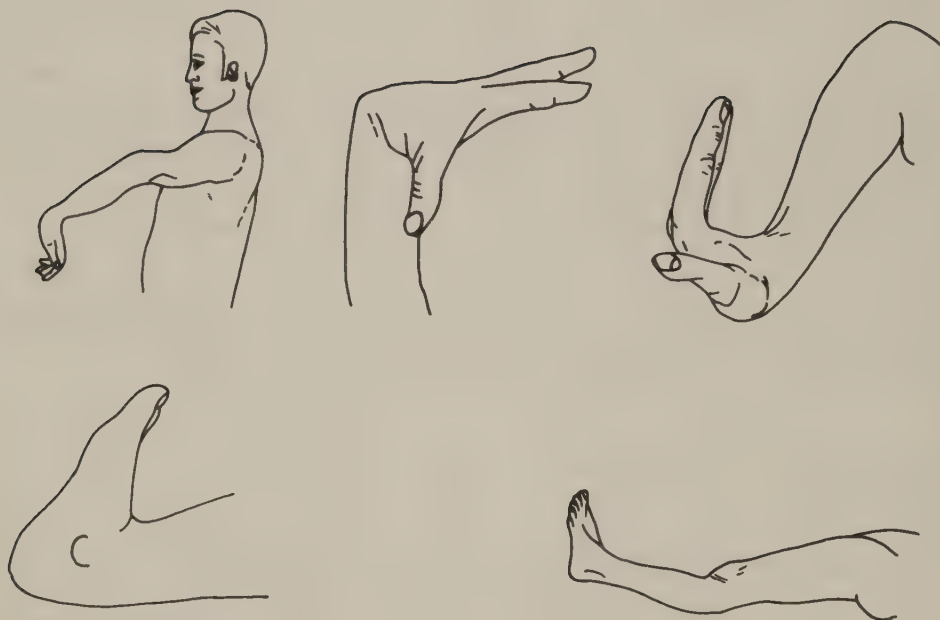


Figure III:1. Hypermobility of the joints was assumed by five variables. It was estimated whether the patients could a) hyperextend the elbow joint more than 10° , b) hyperextend the thumb and flex the wrist until the thumb touched the forearm, c) dorsiflex the fingers and the wrist to make the fingers parallel to the forearm, d) hyperextend the ankle joint more than 45° , e) hyperextend the knee more than 10° .

Coagulation analyses

The following determinations were made:

Coagulation time in glass- and plastic-tubes, recalcification time, prothrombin consumption, bleeding time according to Duke, platelet count, platelet adhesiveness in whole blood according to the method of Hellem and according to the method of Salzman,

AHF (factor VIII), haemophilia B-factor (factor IX), haemophilia C-factor (factor XI), Hageman-factor (factor XII), one-stage prothrombin time, Owren's P&P (prothrombin + factor VII + factor X), factor V, fibrinogen, fibrinolytic activity of plasma and resuspended euglobulin precipitate, on unheated fibrin plates, euglobulin clot lysis time, plasminogen by an immunological method, and fibrin degradation products (FDP) (Nilsson 1974).

The bleeding time was determined according to Ivy, as modified by Nilsson et al. (1963). A cuff was wrapped round the upper arm and inflated to a pressure of 40 mmHg. About 30 to 60 seconds later two transverse incisions were made just below the elbow fold. The incisions were made either with the blade of a surgical knife (Swan Morton No. 11) or with a disposable Simplate IITM. The incisions were 1 mm deep and 10 mm long when made with a blade and 5 mm when made with a Simplate. The blood drops were absorbed with filter paper. The mean of the bleeding times of the two incisions was taken as the bleeding time. The reference values for both methods were the same, 6 - 12 minutes.

Patients about to undergo microsurgery served as controls for the Ivy bleeding time. They had no known bleeding diathesis and had not taken ASA for 10 days before the test. The statistical significance of differences found was checked with Student's t-test.

CASE REPORTS AND RESULTS

The material consisted of cases of EDS known to the Coagulation laboratory or the Orthopedic Department of Malmö General Hospital. There were seven patients from three families.

Family I

In this family there were affected members in five generations (see pedigree in Figure III:2). Three of the affected members were studied, viz. a 55-year-old woman, her son, aged 38, and her daughter, aged 20.

Judging from the clinical features, all the affected members of the first family had classical EDS of type I. They all bruised readily and often had spontaneous subcutaneous haematoma. The son also had recurrent bleeding in the prepatellar bursa. The skin was

hyperextensible, but not fragile. There were typical cigarette- paper thin scars on the knees, elbows and forehead. All three had epicanthus. The joints were hypermobile or had been before the onset of osteoarthritis and/or surgical stabilization. The score for joint hypermobility of the daughter was maximal (5). The son still had hypermobile elbows and knees; the mother, hypermobile knees.

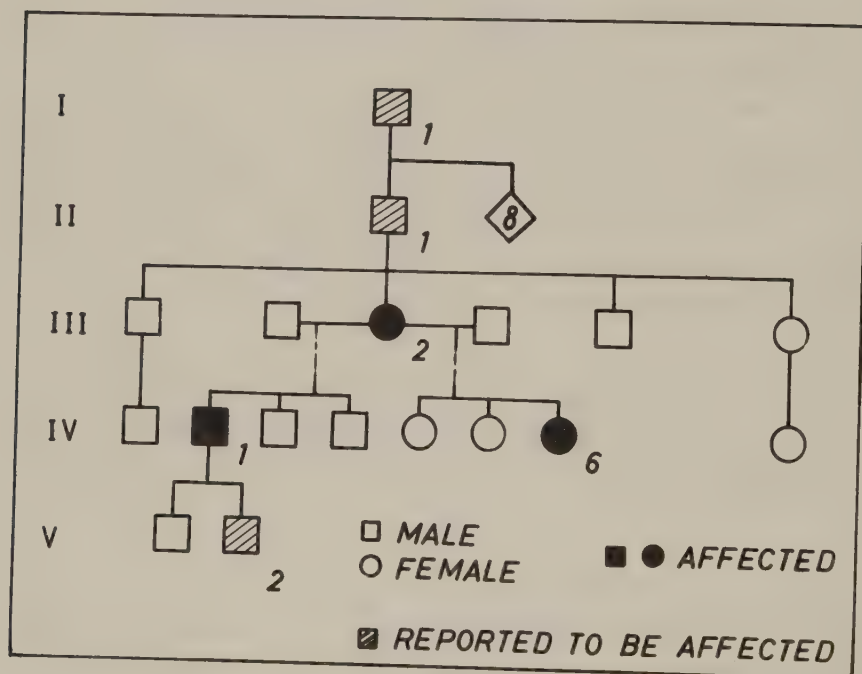


Figure III:2. Pedigree of a family with Ehlers-Danlos syndrome type I.

I: Severe osteoarthritis. Bound to wheelchair. Died from apoplexia at 67 years of age.

II: Died at 39 years of age from rupture of the bowel caused by blunt trauma.

III₂, IV₁ and IV₂ are described in the text.

V: Two years old. Hypermobile joints.

The mother had been operated on for inguinal hernia, varices, twice for tennis elbow, four times for instability and osteoarthritis of her hands (silicon prosthesis of trapezium and an exchange operation, arthrodesis of the cmp joint of the thumbs), triggerfinger of

both hands, carpal-tunnel syndrome of both hands. Four times for hallux valgus, hammer toe and anterior flat feet. Her son had been operated on for inguinal hernia, with cholecystectomy and osteotomy of the proximal tibia because of 60° hyperextension of the left knee. The daughter had been operated on for a tear of the medial meniscus and a loose body in the right knee.

None of these operations had been attended by undue bleeding or complications due to tissue fragility.

The mother and daughter were examined twice at the coagulation laboratory and the son once. Fibrinolysis was not increased. Platelet function was normal and there was no alteration of the coagulation factors that could explain the bleeding diathesis. The bleeding time (Ivy) was normal or just above the upper limit of the normal range (Table III:2). The platelet aggregating power of the collagen of the fascia specimen was normal (Table III:4).

Family II

In the second family two 23-year-old twin brothers were affected. Also their mother, aged 47, was possibly affected, but less so.

The twins had very mobile joints but were not restricted in their daily activities and were regarded as having EDS type III (the benign and hypermobile type). Their grandmother had reportedly strikingly hypermobile joints, but their mother had never considered her joints or skin abnormal. The condition might have been transmitted by a dominant gene with varying penetrance.

The twin brothers often had spontaneous haematomas in the buttocks and spontaneous rupture of small vessels in their fingers once or twice a week. The mother did not bruise readily.

The skin of the twins was very extensible, especially on the dorsal side of the elbows, but it was not fragile. There were no cigarette-paper thin scars. The postoperative scars were broader and thinner than usual but did not differ strikingly from normal. The skin of the mother appeared normal. None of the members of the family had epicanthus. The mother had hypermobile elbows. In the twins the scores for joint hypermobility were

maximal (5).

The mother had been operated on for appendicitis and for varices. She had also been nephrectomized without bleeding complications. One of the twins had been operated on according to Bager for recurrent prepatellar bursitis. Because of recurrent dislocation of his right shoulder he was operated on according to Bankhart. None of the operations had been attended by bleeding complications, but the subscapular tendon was fragile and some stitches cut through the tissue. The late result of the operation was good and no redislocation has occurred (follow-up 2 years).

The results of tests for hemostasis in the mother were normal. The boys showed no increase in fibrinolysis and the coagulation factors were within normal ranges. However, platelet adhesiveness was repeatedly decreased (Table III:2). The bleeding time was normal on all occasions except one (Table III:2). Also the platelet aggregating power of the collagen from fascia specimens was within the normal range (Table III:4).

Family III

In this family there was only one affected member, a 14-year-old girl. Her sister, parents and grandparents were not affected. It was difficult to assign this case to any one of the seven subgroups. The mode of inheritance is not known, but since she was the only affected member in the family, the trait was probably recessive.

Ever since the first year of life she had barely been free from often bulging subcutaneous haematomas. She also suffered from menorrhagia.

The skin was just slightly hyperextensible, but it was fragile and removal of the stitches was sometimes followed by wound disruption. She had typical cigarette-paper thin scars on the knees, elbows and forehead. She also had epicanthus. The joints felt soft and hypermobile, but only the elbows were hypermobile according to the criteria of Carter and Wilkinson. The skin was not thin and, unlike what is seen in Ehlers-Danlos syndrome type IV, the veins were not superficial.

She was operated on for imperforate hymen and for a supracondylar fracture of the right humerus. The tissue was not fragile enough to disturb surgery and there was no bleeding

complication.

Fibrinolysis and the coagulation factors were normal. The platelet adhesiveness was normal on one occasion and slightly subnormal on another (Table III:2). Her bleeding time (Ivy) was slightly prolonged (Table III:2). No fascia specimen was available for testing the platelet aggregating power of the collagen.

Table III:2. Bleeding time and platelet adhesiveness.

Age	Sex	EDS type	Year	Bleeding time according to		Platelet adhesiveness	
				Duke minutes	Ivy minutes	Method of Hellem %	Salzman %
55	F	I	1975	1	9	26	-
			1977	2.5	8 10 14	30	-
38	M	I	1979	-	7	22	-
20	F	I	1972	2.2	17 17	26	21
			1974	2	13	25	-
47	F	III?	1979	1	6	24	-
23	M	III	1971	2	9	9.5	9.5
			1971	2	9	11.5 (15.5; 20.5)	- 23.5
23	M	III	1972	1.5	10	7	7
			1972	1	16	8.5	1.5
15	F	?	1970	4	14 14 14	22	49
			1974	-	13 14	15	-
Normal range				1-5	6-12	17-33	20-60

Table III:3. Bleeding time (Ivy) is longer in patients with Ehlers-Danlos syndrome than in controls (t-test $p < 0.01$).

Number of patients		Bleeding time (Ivy) mts AV $\pm$ SD
EDS	7	10.6 $\pm$ 3.5
Controls	294	8.1 $\pm$ 2.3

Table III:4. The platelet aggregating power (CC%) of collagen from patients with Ehlers-Danlos syndrome does not differ from that of collagen from controls. The amounts of collagen in suspensions made from fascia are also equal.

<u>Diagnosis</u>	<u>Age</u> Yrs	<u>Sex</u> M/F	<u>Collagen</u> mg/l	<u>CC%</u>
EDS I	55	F	10	80
EDS I	37	M	50	260
EDS I	20	F	20	110
EDS III	47	F	70	160
EDS III	23	M	50	160
	AV $\pm$ SD		AV $\pm$ SD	AV $\pm$ SD
EDS (5)	36 $\pm$ 15	2/3	40 $\pm$ 24	154 $\pm$ 68
Controls (15)	42 $\pm$ 10	7/8	37 $\pm$ 14	130 $\pm$ 99

DISCUSSION

Joint instability and foot deformities are common in Ehlers-Danlos syndrome, as are effusions in joints and bursae. Many of the patients develop osteoarthritis (Beighton and Horan 1969a). This means that a patient may often require more than one orthopaedic operation. Moreover, the friability of the tissues and an increased tendency to bleeding increases the risk of complications. In addition, owing to the relatively wide range of interindividual variation, it is not always easy to assess the risks of operation. However, the classification described in the Introduction might prove helpful. Patients in group IV

and certain patients in group I are high surgical risks (Beighton and Horan 1969b). Sometimes, however, it is difficult to assign a given patient to any particular group (Stewart et al. 1977). This was the case with the girl without known heredity in our material.

On surgical incision the skin and deeper tissues of patients most severely affected may cut like cold porridge or "blotting paper". It may be extremely difficult to judge the size of an incision and the wound may widen spontaneously (Beighton and Horan 1969b). Slow healing with wound dehiscence requiring resuture has been described. None of our patients had such friability of the connective tissue, not even those with type I of the disease. There are thus differences also between patients with the same variant, but the severity of the condition and the stigmata are often uniform in a given family (Beighton et al. 1969). This implies that the reports of earlier operations on other affected members of a family may be helpful in preoperative evaluation of a given case.

The bleeding diathesis, which can be more or less severe, is not properly understood. There are several reports of cases with defects of the hemostatic system, but no consistent abnormality, and the defects have been regarded simply as coincidental (Beighton et al. 1969, McKusick 1972). However, the tourniquet test is often positive, which may be related to fragility of the small vessels (Frick and Krafchuck 1956, Barabas and Barabas 1967, Hathaway 1971). Of our patients the twin brothers had decreased platelet adhesiveness, a finding which might simply be coincidental, but which may have added to the hemostatic defect and worsened the bleeding symptoms, which are otherwise often minimal in type III.

The subendothelial collagen initiates hemostasis and the bleeding time can be prolonged in patients with defective collagen (Caen et al. 1970). A correlation between bleeding time and the platelet aggregating power of the collagen has also been described (Udén and Nilsson 1979). It was therefore thought that in EDS the platelet aggregating power of collagen might be decreased. But this proved not to be the case, possibly because the patients were not severely affected, although some of them belonged to type I. Another possibility is that the basic defect in these variants is not at the molecular level, but at a higher level of organization of the collagen fibres. A decreased platelet aggregating power of a fascia preparation has been described in two cases of EDS (Karaca et al. 1972). One

of these patients had severe bleeding symptoms as well as a prolonged Ivy bleeding time (> 30 min.), but a normal bleeding time according to Duke.

The bleeding time in EDS has been described as normal (Frick and Krafchuck 1956, Hathaway 1971). However, we used the method of Ivy, as modified by Nilsson et al. (1963). This modification makes the test more sensitive. With the use of a new device (Simplate IITM) the method is easy to standardize without loss of its sensibility (Nilsson et al. 1979). The method can detect minor defects in the hemostatic system, such as that in von Willebrand's disease, in which mild cases often have a normal bleeding time according to Duke. With this modification of Ivy's method we were able to demonstrate a slight prolongation of the bleeding time in the group of patients with EDS (Table III:3). There was no undue bleeding at surgery; nor was any such loss expected for the bleeding time was only slightly prolonged. In more severe cases of EDS this method may be sensitive enough to detect patients at risk, such as the boy described by Karaca et al. (1972).

Summing up, experience of earlier operations on the patient as well as on affected relatives is important for detecting patients at risk. Also knowledge of the variant of the disease to which a given case belongs may be helpful. A preoperative examination for coincidental coagulation and bleeding disorders is recommended since many defects might be substituted for or treated during an operation. There is also some evidence that the modification of Ivy's method will be sensitive enough to offer some guidance. If the bleeding time proves prolonged, it would at least be of theoretical interest to estimate the platelet aggregating power of the collagen.

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PAPER IV

COLLAGEN-INDUCED PLATELET AGGREGATION AND BLEEDING TIME IN ADOLESCENT IDIOPATHIC SCOLIOSIS

ABSTRACT

Collagen is the main supportive protein of connective tissue. Another function is the initiation of hemostasis by activation of the platelets. It has been suggested that collagen is abnormal in idiopathic scoliosis. The present study lends further support to this view, it having revealed that collagen of fascia specimens from patients with adolescent idiopathic scoliosis aggregates platelets less readily than collagen from non-scoliotic controls and that also suspension of fascia from patients with adolescent idiopathic scoliosis contains more collagen than such suspension from the controls. The collagen abnormality is also probably reflected in a prolonged bleeding time. The changes in the collagen in patients with scoliosis persist at least some years after the cessation of growth.

INTRODUCTION

Of the possible underlying causes of idiopathic scoliosis, only hereditary factors are now widely accepted, but nothing definite is known of their mode of action (Wynne-Davies 1968, Cowell et al. 1972, Harrington 1977). In the last decade a disturbance of connective tissue has been discussed as a possible causal factor, it having been observed that scoliosis is unusually common in patients with defective collagen e.g. in Ehlers-Danlos syndrome and in Marfan's syndrome (McKusick 1972).

Collagen is the most important supportive factor of connective tissue. Another important function of collagen is the initiation of hemostasis by activation of the platelets (Hugues 1960). The connection between collagen defects and impaired hemostasis has been described in different types of Ehlers-Danlos syndrome (Karaca et al. 1972, McKusick 1972,

Pinnell et al. 1972) and in scurvy (Caen and Legrand 1972).

This paper concerns possible defects of the collagen in adolescent idiopathic scoliosis and the influence of the collagen on the bleeding time.

MATERIAL

The material consisted of 14 females and 1 male with adolescent idiopathic scoliosis with a range of the curves between $50 - 100^{\circ}$ according to Cobb (1948). The patients were operated on in a one step procedure with correction and internal fixation according to Harrington and posterior fusion. Biopsy specimens were obtained from the fascia of the longitudinal dorsal muscles on the convex as well as on the concave side of the curve. Ten age-matched patients (three females and seven males) operated on for disc herniation, spondylolisthesis or fractures of the extremities served as controls (Table IV:1). In five cases the specimens were obtained from the same part of fascia as in the scoliotic group; in the remaining five, from muscle fasciae of the extremities.

The bleeding time was determined in 68 patients, aged 11 to 32 years (mean 18 ± 6 years) operated on for scoliosis, and in 24 healthy non-scoliotic controls, aged 11 to 16 years (mean 13 ± 2 years).

METHODS

Collagen suspensions were prepared as described in detail elsewhere (Udén and Nilsson 1978). In short the pieces of fascia were scraped clean, freeze-dried in vacuum and homogenized in icecold 8.35 mM acetic acid for 60-90 seconds. The suspension was stored at -60°C . Before being used it was rehomogenized and centrifuged at 1200 g for 30 minutes at $+4^{\circ}\text{C}$. The supernatant, which was clear, was immediately used to determine the platelet aggregating power of the collagen. The amount of collagen in the supernatant was calculated from the content of hydroxyproline measured by a modified Woessner method (Udén and Nilsson 1978).

The platelet aggregation was assessed photometrically. A Payton Dual Channel Aggregation Module was used. It includes constant magnetic stirring and constant recording of the transmission. The lowest concentration of collagen in platelet-rich plasma (PRP) that induced platelet aggregation was called the critical concentration (CC) - high CC is associated with low aggregating power (Udén and Nilsson 1978). PRP was obtained from healthy volunteers. Since the responsiveness of PRP even from healthy persons varies, the CC of the collagen suspensions from scoliotic patients and from controls were also expressed in relation to that of a standard collagen (CC%), which is prepared from a slice of fascia with known platelet aggregating power.

The bleeding time was determined according to Ivy (Nilsson et al. 1963).

The statistical methods used were Student's t-test for differences between means and Pearson's linear correlation test.

RESULTS

The collagen content of the suspension from patients with scoliosis was significantly higher than that from controls, and the collagen from patients with scoliosis also aggregated platelet only one fourth as efficiently as collagen from the controls (Table IV:1). The first figure in brackets (Table IV:1) denotes the mean value found for the control samples obtained from the same back muscle fascia as in the patients with scoliosis; the second figure, the corresponding figure for the other controls.

Platelet aggregation and the concentration of collagen in the suspension did not vary with the patients' age ($r = -0.2$ and 0.07 , respectively). However, in the scoliotic group a significant correlation ($p < 0.01$) was found between CC and collagen concentration in the suspension ($r = 0.68$), which means that the more easily the collagen was dissolved the less it aggregated platelets. There was no significant difference in collagen content in the suspensions or platelet aggregation properties with sex.

The Ivy bleeding time in 68 patients with scoliosis was 9.6 ± 2.6 (AV $\pm$ SD) minutes and 7.9 ± 1.7 (AV $\pm$ SD) minutes in the controls. The difference was significant ($p < 0.01$).

Table IV:1. Results of the analysis of collagen from patients with idiopathic scoliosis and controls. The first figure in brackets denotes the mean values for control samples obtained from back muscle fascia; the second figure, mean values for the other control samples. CC = minimal concentration of collagen in platelet-rich plasma (PRP) that induces platelet aggregation.

	Number	Age years		Collagen in suspension mg/l		CC mg collagen/l PRP		CC % of standard AV + SD
		AV	SD	AV	SD	Geometric mean and range	AV + SD	
Scoliosis	15	18	4	130	70	2.5 (1.3 - 8.4)	3.2 + 2.4	770 + 550
Controls	10 (5 + 5)	19	6	550	20 (50 50)	0.7 (0.2 - 1.4)	0.8 + 0.4 (1.0 0.6)	180 + 110 (250 110)
t-test for difference between means				p < 0.01		p < 0.001	p < 0.01	p < 0.01

There was no correlation between age and bleeding time ($r = 0.009$).

DISCUSSION

Collagen is the only constituent of connective tissue that has any appreciable tensile strength (Grant and Prockop 1972 a), and defects of the collagen can cause scoliosis. For instance, scoliosis can be provoked in animals by β -amino-propionitrile (Ponseti and Baird 1952), which inhibits the first reaction necessary for inter-molecular cross linking of collagen (Grant and Prockop 1972 b). Also in a highly inbred line of chickens with a readily soluble collagen there was an incidence of scoliosis of 55% (Riggins et al. 1977).

Also in idiopathic scoliosis collagen abnormalities are on record. Francis et al. (1977) tested the resistance of extracted polymeric skin collagen against depolymerisation by Na OH. This resistance was decreased in most patients with adolescent idiopathic scoliosis as compared with non-scoliotic controls. The authors felt that a decrease in polymeric collagen resistance reflects a corresponding decrease in vivo of the number of other types of collagen cross-links. The abnormality was greatest in the youngest patients.

It has been reported from Australia that changes in the collagen content of scoliotic discs are related to the degree of scoliosis and disc mobility (Taylor et al. 1976). To distinguish between primary and secondary effects the authors tested the extractability of collagen by pepsin across thirteen zones of normal and scoliotic discs and found a significant difference between patients with scoliosis and the controls. The values did not vary with the side of the curve from which a given specimen had been obtained and could thus not be related to mechanical loading (Bushell et al. 1978).

Other authors have found no alterations in collagen from patients with idiopathic scoliosis, for instance, Nordwall compared tendons and ligaments from 40 patients with idiopathic scoliosis with corresponding specimens from 20 patients with paralytic or congenital scoliosis. He tested the biomechanical properties, the shrinkage on heat denaturation and the collagen content. He found no significant difference between the groups (Nordwall 1973).

Bradford et al. (1977) studied skin fibroblasts in culture. They compared patients with

idiopathic scoliosis with seven age-matched controls and found no significant difference in any parameter tested (cell layer, extractability with salt or acetic acid and hydroxy-proline to proline ratio) except that the fibroblasts from the scoliotic patients had a lower hydroxy-proline to proline ratio in the non-dialyzable media (Bradford et al. 1977). The authors concluded that idiopathic scoliosis is not a generalized connective tissue disorder.

A perusal of the literature failed to reveal any studies of the platelet aggregating properties of collagen from patients with idiopathic scoliosis. Our finding that the collagen in adolescent idiopathic scoliosis had a decreased platelet aggregating power might be an artefact caused by the preparation of the collagen suspensions, for instance, interaction of the state of aggregation of the collagen itself due to storing. However, the suspensions were all used within two hours and a correction for changes with time is included in the method (Udén and Nilsson 1978).

We used a crude suspension of collagen in the test of the platelet aggregating power. It was because the collagen otherwise loses most of its aggregating power (Udén and Nilsson 1978). Using a suspension one can also get information of the insoluble part of the collagen, though it is then not possible to assess the percentage of the total collagen that is solubilized, but it was clear that the suspension of fascia from patients with adolescent idiopathic scoliosis contained more collagen than corresponding suspensions from non-scoliotic controls and that the collagen had a decreased platelet aggregating power in vitro. A possible explanation of these findings is an altered cross-linking of the collagen as in Ehlers-Danlos syndrome type VI, which is characterized by scoliosis and a bleeding tendency and in which the basic defect is a decreased cross-linking due to lack of lysin-hydroxylase (Pinnell et al. 1972). Another possibility is a decreased content of collagen type III, which has an increased platelet aggregating power (Balleisen et al. 1975), or an otherwise altered structure of the collagen. However, the main point is that there are significant differences between the scoliotic fascia, which speak in favour of collagen being involved in the development of idiopathic scoliosis.

The prolonged bleeding time also supports the concept of an in vivo defect of the platelet aggregating power of the collagen. The bleeding time was not so prolonged as to cause any undue bleeding during the operation.

Inter-individual comparison of collagen-induced platelet aggregation is best based on identical anatomical structures (Udén and Nilsson 1978). In five of our controls this was not the case. However, they did not differ much from the other controls, and the differences between the patients with scoliosis and the two subgroups was still significant ($p < 0.05$). About one third of the patients with scoliosis were adults and since they did not demonstrably differ from the younger patients it would appear that the collagen defect persists even in adult age.

The methods used in this paper and in the above cited papers give indirect information about the mature collagen and may reflect different qualities of this complex molecule. It is therefore not astonishing that abnormalities are found in some investigations but not in others. For instance, a defect of the collagen due to some hormonal influence or an increased rate of synthesis is not necessarily observable in a fibroblast culture.

Our knowledge of the role played by collagen in scoliosis is still very limited and the basic defect or defects are not known, but, judging from our present knowledge, it seems likely that collagen is at least involved in the development in some cases of adolescent idiopathic scoliosis.

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PAPER V

COLLAGEN CHANGES IN CONGENITAL AND IDIOPATHIC SCOLIOSIS

ABSTRACT

Collagen abnormalities in adolescent idiopathic scoliosis have been recorded. In this condition the platelet aggregating power of collagen is decreased and the amount of collagen in suspensions prepared from fascia is increased. We found the same abnormalities in two out of four patients with congenital scoliosis, operated on according to Harrington. In idiopathic as well as in congenital scoliosis the decrease in the platelet aggregating power was more conspicuous in the younger patients.

INTRODUCTION

Collagen, the most important supportive protein of connective tissue, has been shown to have defective properties in adolescent idiopathic scoliosis (AIS) (Francis et al. 1977, Bushell et al. 1978, Udén et al. 1979). In AIS the solubility of collagen in weak acetic acid is increased and the platelet aggregating power is diminished (Udén et al. 1979). These changes can be explained by an altered cross-linking, or by a decreased content of type III collagen, which is less soluble and has an increased platelet aggregating activity (Balleisen et al. 1975). This paper compares collagen in congenital scoliosis with that in AIS.

MATERIAL

The material consisted of four patients (15^{+3} years) with congenital scoliosis (Table V:1) and eight patients (15^{+2} years) with AIS. The scoliosis curvature ranged from 47° - 80° and 51° - 61° , respectively. All the patients with congenital scoliosis had a unilateral bar on the concave side at the apex of the curvature. Two patients also had several hemivertebrae.

Table V:1. Four cases of congenital scoliosis. CC% = minimal concentration of collagen in platelet-rich plasma (PRP) that induces platelet aggregation, relative to that of a standard collagen.

Sex	Age years	Defect of the vertebrae	Collagen in in suspension mg/l	CC% of standard
M	17	Unilateral bar + hemivertebrae	140	770
M	18	Unilateral bar	25	60
F	14	Unilateral bar	85	250
F	11	Unilateral bar + hemivertebrae	120	1080

The patients were operated upon according to Harrington, and biopsy specimens were obtained from fascia of the longitudinal dorsal muscles.

METHODS

Collagen suspensions were prepared as described in detail elsewhere (Udén and Nilsson 1978). The pieces of fascia were scraped clean and freeze-dried in a vacuum, 8.35 mM acetic acid was added in an amount corresponding to 50 times the weight of the specimen, after which the mixture was homogenized for 60-90 seconds with a pestle and a glass mortar. An equal volume of distilled water was then added and homogenization was repeated. During the homogenization the mortar was kept in ice-cold water. The suspension was stored at -60°C . Before being used it was rehomogenized and centrifuged at 1200 g for 30 minutes at $+4^{\circ}\text{C}$. After centrifugation the amount of collagen in the supernatant was calculated from the content of hydroxyproline measured by a modified Woessner method. The supernatant was used to determine the platelet aggregating ability of the collagen.

The platelet aggregating ability was assessed photometrically with a Payton Dual Channel Aggregating Module, also described by Udén and Nilsson (1978). It implies constant magnetic stirring and constant recording of the transmission. The lowest concentration of collagen in platelet-rich plasma (PRP) that induced platelet aggregation was called the

critical concentration (CC). PRP was obtained from healthy volunteers. Since the responsiveness of PRP even from healthy persons varies, the CC was expressed in relation to that of standard collagen (CC%).

In the statistical treatment of the data, Student's t-test for differences between means, and Pearson's linear correlation test were used.

RESULTS

The mean collagen concentrations in the suspensions from the two groups were equal, as was the mean CC%, which reflects the platelet aggregating power (Table V:2).

Compared with the findings in non-scoliotic controls (Udén et al. 1979) the concentration of collagen in the suspensions was significantly increased in congenital scoliosis ($p < 0.05$) as well as in idiopathic scoliosis ($p < 0.05$). In neither of the scoliotic groups did the platelet aggregating power of the collagen (CC%) differ significantly from the control value but a difference was found when the two groups were pooled ($p < 0.01$). This implies a decreased platelet aggregating power of the collagen of the scoliosis patients regardless of type.

The CC% varied inversely with the patient's age, ($r_{12} = -0.69$) which means that the collagen of the younger patients had a more reduced platelet aggregating power.

There was also a slight though not significant correlation between age and collagen content of the solution ($r_{12} = -0.46$).

The material, five boys and seven girls, showed no significant sex differences in either collagen content of the suspensions (86^{+43} mg/l and 95^{+39} mg/l, respectively) or the mean CC% values ($340^{+290}\%$ and $540^{+400}\%$, respectively). The values in the girls were not significantly higher than those in the boys.

Table V.2. Results of the analysis of collagen from patients with congenital and idiopathic scoliosis (AIS). The figures for AIS II and controls are from an earlier paper (Udén et al. 1979). CC% = minimal concentration of collagen in platelet-rich plasma (PRP) that induces platelet aggregation, relative to that of a standard collagen.

	Number	Sex		Age years	Collagen in suspension mg/l		CC% of standard	
		Male	Female		AV	SD	AV	SD
Congenital scoliosis	4	2	2	15 ⁺ - 3	90 ⁺ - 50	540 ⁺ - 470		60-1080
AIS I	8	3	5	15 ⁺ - 2	90 ⁺ - 30	420 ⁺ - 310		140-1130
AIS II	15	1	14	18 ⁺ - 4	130 ⁺ - 20	770 ⁺ - 550		150-2050
Controls	10	7	3	19 ⁺ - 6	50 ⁺ - 20	180 ⁺ - 110		60-400

DISCUSSION

Scoliosis can be induced by inhibition of the naturally occurring cross-links of collagen, as described by Ponseti and Baird (1952), who induced scoliosis in rats with beta-amino propionitrile. Scoliosis has also been seen in 55% of a highly inbred strain of chickens with a collagen defect, characterized by an increased solubility in 4 M urea (Riggins et al. 1977). When it was also found that the properties of collagen in adolescent idiopathic scoliosis (AIS) differed from the normal, it was suspected that defective collagen was the cause of AIS. If congenital scoliosis is caused solely by anomalies of the vertebrae, one would not expect the same collagen defect in such cases. However, we found the same kind of changes as in AIS, i. e. an increased solubility in weak acetic acid and a decreased platelet aggregating power.

Congenital scoliosis is a rare condition - collagen samples could be obtained from only four cases. Consequently, the value of the statistical methods is limited. However, in two of the cases of congenital scoliosis the CC% values were well above the upper limit of the normal range and more on the level of that in AIS (Tables V:1 and V:2). There is no evidence that the collagen defect in congenital scoliosis is the same as in idiopathic scoliosis. However, two of the cases showed signs of a similar abnormality. This was perhaps because all our fascia specimens were obtained from severe cases. Only relatively few cases of congenital scoliosis, as well as idiopathic scoliosis, deteriorate to this degree during adolescence. Possibly such severe deformities require some co-existing disturbance of the collagen. Any test of this hypothesis would require inclusion of cases with mild deformities.

The sex ratio was not the same in the two groups. However, the variation of the properties of collagen with sex was not statistically significant and cannot explain the difference found between the patients with scoliosis and the controls.

The correlation between age and platelet aggregating properties of the collagen has been observed earlier by Udén et al. (1979). But in that study it was not statistically significant. The finding that this abnormality is more pronounced in younger patients is in agreement with Francis et al. (1977).

It is known that the amount of reducible cross-links of collagen varies with the growth rate (Bailey et al. 1974). Perhaps this is one cause of the frequently observed progression of both congenital and idiopathic scoliosis during the prepuberal growth spurt (Farkas 1954, Shands and Bundens 1956, Duval-Beaupère 1971, Clarisse 1974).

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PAPER VI

BLEEDING TIME AND SCOLIOSIS

ABSTRACT

Collagen abnormalities in idiopathic scoliosis are on record. Since there is a correlation between the platelet aggregating power of the collagen and the bleeding time in patients with scoliosis, the bleeding time was studied in 195 cases with scoliosis and in 318 controls. The bleeding time was longer in the females, especially in those with idiopathic scoliosis ($n = 149$). Patients with paralytic scoliosis ($n = 5$) also had a significantly longer bleeding time than non-scoliotic controls. The patients with congenital scoliosis ($n = 13$) did not differ significantly from the controls or from patients with idiopathic scoliosis. The bleeding time in idiopathic scoliosis did not vary with age or magnitude of the scoliosis. Our data support a view that collagen abnormalities play a role not only in the etiology of idiopathic scoliosis, but also in other forms of scoliosis.

INTRODUCTION

Collagen is responsible for most of the tensile strength of the connective tissue. It also plays an important role in normal hemostasis. When a vessel wall is damaged platelets adhere to the exposed subendothelial collagen, and this contact initiates a chain of reactions resulting in the formation of a platelet thrombus (Gordon 1979).

Collagen abnormalities have been described in idiopathic scoliosis (Francis et al. 1977, Bushell et al. 1978, Uden et al. 1980a). In adolescent idiopathic scoliosis the platelet aggregating power of collagen is decreased and the bleeding time prolonged (Udén et al. 1980a). Similar abnormalities are sometimes seen also in congenital scoliosis (Udén et al. 1980b). These observations have been made in collagen of fascia specimens obtained at surgery of patients because of moderate or severe scoliosis ($> 45^{\circ}$). We do not think it

ethical to obtain biopsy specimens of fascia from patients with only mild scoliosis not requiring surgery. Therefore, nothing is known about the collagen in such cases. However, there is a correlation between the platelet aggregating power of the collagen and the bleeding time in scoliosis (Udén and Nilsson 1979). It was therefore decided to study the bleeding time in idiopathic, congenital and paralytic scoliosis with respect to sex, age and magnitude of the scoliosis.

MATERIAL

The material consisted of 108 patients operated on for scoliosis at the Department of Orthopedic Surgery at Malmö General hospital and 86 patients who were treated conservatively with a brace or only expectantly. The material is summarized in Table VI:1.

Table VI:1. Age and sex distribution of patients with scoliosis.

Type	Males		Females	
		Years AV $\pm$ SD		Years AV $\pm$ SD
Idiopathic				
operated on	(13)	18 $\pm$ 4	(79)	18 $\pm$ 4
not operated on	(14)	16 $\pm$ 2	(70)	14 $\pm$ 2
Congenital				
operated on	(5)	17 $\pm$ 1	(6)	14 $\pm$ 3
not operated on	-	-	(2)	13 $\pm$ 1
Paralytic				
operated on	(3)	19 $\pm$ 1	(2)	24 $\pm$ 12
not operated on	-	-	-	-

Figures within brackets indicate number of patients.

The patients with congenital scoliosis had an unilateral bar on the concave side and/or one or several hemivertebrae. Of the five patients with paralytic scoliosis two had cerebral palsy, one had had poliomyelitis, and one had the progressive neuromuscular atrophy of Charcot-Marie-Tooth and one suddenly got paraplegia (anterior spinal artery

syndrome) at 13 years of age. Twenty-four healthy volunteers and 357 patients without known bleeding diathesis and tested at the Coagulation Laboratory, Malmö General Hospital, prior to microsurgery served as controls (Table VI:2). Since the mean age of the controls was higher than in the groups with scoliosis and since only few of the patients with scoliosis were older than 25 years, the controls were divided into two groups, one below 26 years and one 26 years or above.

Table VI:2. Age and sex distribution of the controls.

	Males		Females	
	Years		Years	
	AV $\pm$ SD		AV $\pm$ SD	
Controls (167)	41 $\pm$ 19	(151)	39 $\pm$ 19	
$\leq$ 25 years (37)	14 $\pm$ 5	(46)	15 $\pm$ 5	
$\geq$ 26 years (130)	49 $\pm$ 14	(105)	49 $\pm$ 13	

Figures within brackets denote number of patients.

METHODS

The following determinations were made in the 108 patients operated on for scoliosis.

Coagulation time in glass- and plastic-tubes, recalcification time, bleeding time according to Duke and Ivy, platelet count, platelet adhesiveness in whole blood according to the method of Hellem and according to the method of Salzman, factor VIII, factor IX, factor XI, factor XII, one-stage prothrombin time, Owren's P&P test (prothrombin factor VII and factor X), factor V, fibrinogen, fibrinolytic activity of plasma and resuspended euglobulin precipitate on unheated fibrin plates, euglobulin clot lysis time, plasminogen by an immunological method, and fibrin degradation products (FDP) (Nilsson 1974).

In all the patients and the controls the bleeding time was determined according to Ivy, as modified by Borchgrevink and Waaler (1958) and Nilsson et al. (1963). A cuff was wrapped round the upper arm and inflated to a pressure of 40 mm Hg. About 30 to 60 seconds later two transverse incisions were made just below the elbow fold. The incisions

were made either with the blade of a surgical knife (Swan Morton No.11) or with a disposable Simplate IITM. The incisions were 1 mm deep and 10 mm long when made with a blade and 5 mm when made with a Simplate IITM. The blood was blotted every 30 seconds as long as bleeding continued. The mean of the bleeding times after the two incisions was taken as the bleeding time. The values found did not vary with the technique used. Thus, in 225 patients the surgical blade technique gave a bleeding time of 7.75 ± 2.26 (AV $\pm$ SD) and the Simplate IITM technique 7.69 ± 2.40 (AV $\pm$ SD) (Nilsson et al. 1979).

Statistical methods used were: Student's t-test for checking the significance of differences between means and Pearson's correlation test.

RESULTS

The bleeding time in the different groups with scoliosis and in the controls is given in Table VI:3. In all the groups the mean bleeding time was longer in the females. But the difference was statistically significant only in the group of idiopathic scoliosis ($p < 0.01$) and if all the females were pooled and compared with all the males ($p < 0.001$). There was no significant correlations between age and bleeding time, neither in the controls nor in the group with idiopathic scoliosis (Figure VI:1). Thus the coefficient of correlation was -0.08 in the male controls ($n = 167$) and -0.02 in the females ($n = 151$); in the males with idiopathic scoliosis 0.32 ($n = 27$) and in the females 0.14 ($n = 149$). However, since the bleeding time was somewhat longer though not significantly in controls below 26 years than in controls 26 years or older (Table VI:3), we also compared the scoliosis groups with this age-matched control group.

The mean bleeding time in the females with idiopathic scoliosis was longer than in the female controls ($p < 0.001$). It was also longer than in the age-matched females controls ($p < 0.005$). However, the males with idiopathic scoliosis did not differ significantly from the male controls.

Table VI:3. Ivy bleeding time in patients with scoliosis and controls.

	Males		Females		Total	
		AV $\pm$ SD		AV $\pm$ SD		AV $\pm$ SD
Idiopathic scoliosis	(27)	8.5 $\pm$ 2.0	(149)	10.0 $\pm$ 2.8	(176)	9.8 $\pm$ 2.8
Congenital scoliosis	(5)	7.5 $\pm$ 1.6	(8)	9.2 $\pm$ 2.2	(13)	8.5 $\pm$ 2.1
Paralytic scoliosis	(3)	10.3 $\pm$ 3.2	(2)	12.0 $\pm$ 1.0	(5)	11.0 $\pm$ 2.5
Controls $\leq$ 25 years	(37)	8.3 $\pm$ 1.9	(46)	8.7 $\pm$ 2.0	(82)	8.5 $\pm$ 2.0
Controls $\geq$ 25 years	(130)	7.8 $\pm$ 2.4	(105)	8.2 $\pm$ 2.2	(236)	8.0 $\pm$ 2.3
Controls total	(167)	7.9 $\pm$ 2.3	(151)	8.3 $\pm$ 2.3	(318)	8.1 $\pm$ 2.3

Figures within brackets denote the number of patients or controls.

The mean bleeding time in patients with congenital scoliosis did not differ significantly from that in the controls or from that in patients with idiopathic scoliosis. The mean bleeding time in the five patients with paralytic scoliosis was significantly longer than that in the controls ($p < 0.01$). Compared with that in the age-matched controls the difference was also significant ($p < 0.02$). No correlation was found between age at onset of the scoliosis and the bleeding time (Figure VI:2). Neither was any correlation found between the bleeding time and the magnitude of the scoliosis in the idiopathic cases (males $r = 0.29$, $n = 27$, females $r = -0.15$, $n = 149$). This is also illustrated in Figure VI:3.

Of 79 females operated on for idiopathic scoliosis, only 16 showed minor abnormalities in the coagulation and bleeding tests. The fibrinolytic activity was slightly to moderately increased in 12 of them, and the platelet adhesiveness was slightly decreased in four. In one patient factor VII was low (50%). In this group of patients with minor abnormalities in the hemostatic tests the mean bleeding time was 10.3 ± 3.1 minutes (AV $\pm$ SD). The mean value for the other 63 females was 9.7 ± 2.5 (AV $\pm$ SD). The difference was not statistically significant.

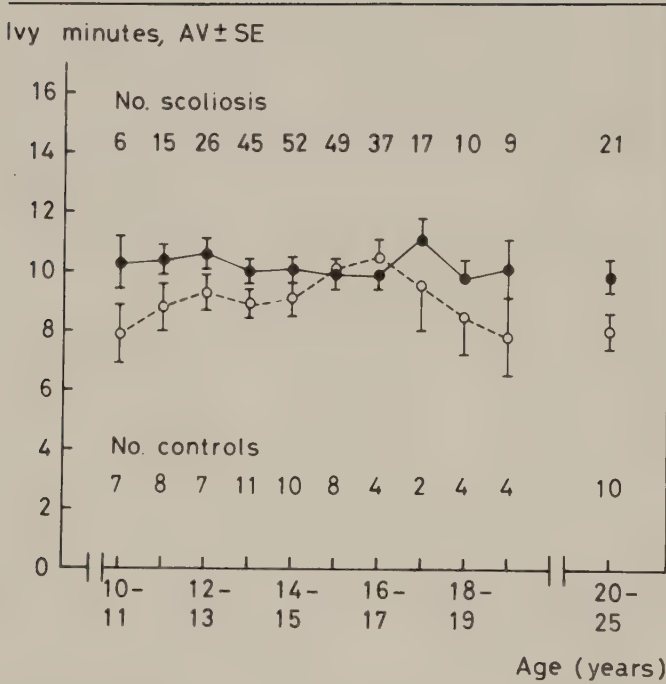


Figure VI:1. Bleeding time (Ivy) in relation to age. Running average and standard error for females with idiopathic scoliosis and female controls. No significant correlation.

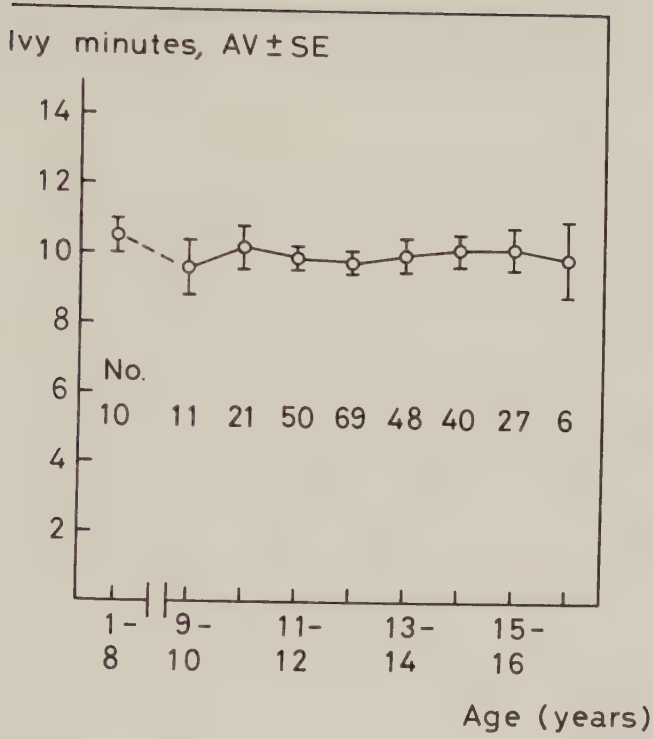


Figure VI:2. Bleeding time (Ivy) in relation to age (CA) at onset of the scoliosis. Running average and standard error for females with idiopathic scoliosis. No correlation between age at onset and bleeding time.

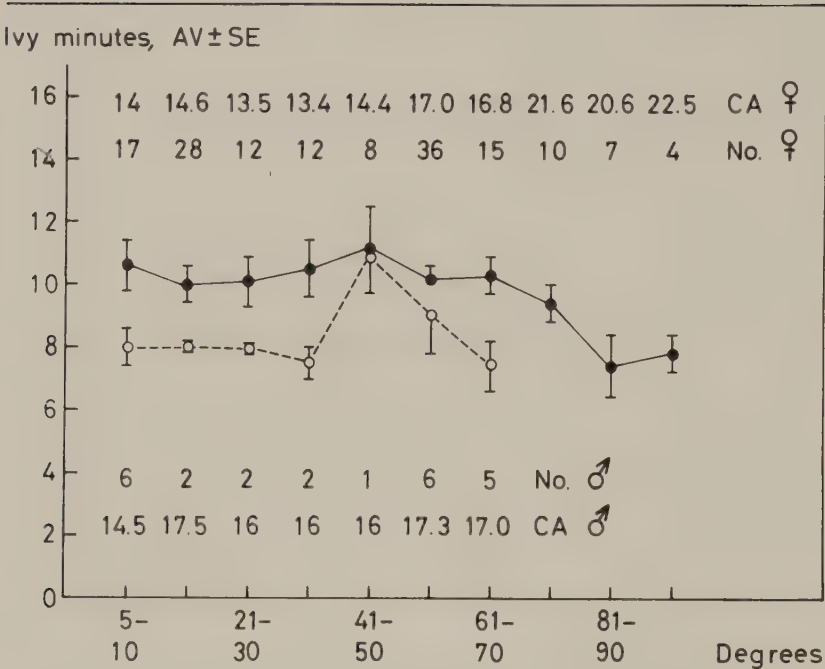


Figure VI.3. Bleeding time (Ivy) in relation to the magnitude of the scoliosis. Average and standard error for male and female with idiopathic scoliosis. No significant correlation between magnitude of the curves and bleeding time.

DISCUSSION

The Ivy bleeding time modified in the way described by Nilsson et al. (1963) is a sensitive test capable of detecting even small abnormalities in primary hemostasis. The mean bleeding time in the patients with scoliosis was longer than in the controls, but the bleeding time in most of the patients with scoliosis was within the normal range, and in the few in whom it was prolonged it was not attended by any detectable increase in blood loss at surgery for scoliosis.

Laboratory studies of the patients operated on for scoliosis did not show any abnormalities, such as von Willebrand's disease or platelet defects, which could otherwise explain the prolonged bleeding time. In some patients with scoliosis the fibrinolytic activity was slightly increased. Such an increase can be due to stress during blood sampling (Brozovic 1977) and a similar increase could also be expected in the controls who were also exposed to the same stress.

A correlation between the platelet aggregating power of the collagen and the bleeding time has been reported in an earlier paper (Udén and Nilsson 1979). It is therefore tempting to assume that the prolonged bleeding time in scoliosis might be caused by abnormalities of the collagen that possibly play a role in several types of scoliosis since the bleeding time was prolonged not only in the females with idiopathic scoliosis but also in the few cases with paralytic scoliosis. In fact, collagen abnormalities have been reported also in congenital scoliosis (Francis et al. 1976, Udén et al. 1980b). One possible explanation of these abnormalities in all three types of scoliosis might be secondary alterations due to an increased turnover of the collagen caused by rebuilding of the connective tissue during the progress of the scoliosis, but since the bleeding time is prolonged also in adults in whom the scoliosis is supposed to be non-progressive, this explanation is not likely. Nor is the bleeding time more prolonged in the severe cases, which it should be if the abnormalities were secondary.

A tempting hypothesis is that the scoliosis can be initiated by other factors, such as disturbed equilibrium in idiopathic scoliosis (Sahlstrand 1977), paralytic muscles, as in paralytic scoliosis, or malformations of the vertebrae, as in congenital scoliosis, and that the defective collagen allows the curvature to progress as proposed by Bushell et al. (1979). Our data do not lend support to the assumption that collagen has a major influence on the progress of scoliosis for no correlation was found between the bleeding time and the severity of the scoliosis, possibly because the bleeding time is too crude as an indicator of collagen abnormalities.

The bleeding time was longer in females although the difference was statistically significant only in idiopathic scoliosis. This sex difference remains unexplained. However, it could be relevant since clinically important scoliosis is more common in females. Again, this is true for idiopathic and congenital cases (Winter et al. 1968, Nordwall 1973), but

not for the paralytic (James 1976).

Genetical studies support a multifactorial etiology of idiopathic scoliosis (Filho and Thompson 1971, Riseborough and Wynne-Davies 1973) and neuromuscular, hormonal and constitutional factors may be involved (Sahlstrand 1977, Willner 1974, Willner et al. 1976). In congenital scoliosis the malformation is of obvious importance and in paralytic scoliosis the paralysis. However, our findings suggest that also collagen abnormalities may play a role in the development not only of idiopathic scoliosis, but also of other forms of scoliosis.

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